THE IMPACT OF INTERFERON GAMMA ON PANCREATIC BETA CELL LIPID METABOLISM AND FUNCTION

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ABSTRACT

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Type 1 diabetes (T1D) is characterized by loss of blood glucose control due to autoimmune attack of insulin secreting β cells within the pancreatic islet. During the immunologic response, proinflammatory cytokines are secreted by immune cells and contribute to β cell loss. Interferon gamma (IFNγ) is an anti-viral cytokine with proinflammatory and immunomodulatory effects, and elicits pleiotropic impacts on β cell function. Current studies have demonstrated a novel role of fatty acid (FA) and cholesterol metabolism in host cell defense against infection. In addition, IFNs have been shown to alter immune cell lipid metabolism that is directly link to activation of immune responses. Currently, there is a lack of understanding of the role of IFNγ on β cell lipid metabolism and whether it is associated with IFNγ-mediated effects on β cell function. Here, *in vivo* study in a T1D-susceptible model (LEW.1WR1 rats) showed that induction of islets autoimmunity with viral mimetic resulted in elevated and sustained IFNγ signaling, concomitant with a significant increase of triacylglyceride (TAG) levels in pancreatic islets. The effects of IFNγ on lipid metabolism therefore was examined in β cell line INS-1. Treatment of INS-1 cells with IFNγ led to a dynamic change in TAG levels and lipid droplets (LD): a decrease at 6 h and an increase at 24 h in TAG levels and LD numbers. Gene expression results suggested that IFNγ transiently induces lipolysis, followed by upregulation of *de novo* lipogenesis (DNL). Importantly, IFNγ potentiated anti-viral gene expression stimulated by viral mimetic, and pharmacological inhibition of DNL abrogated this priming effect by IFNγ, suggesting that IFNγ-induced DNL is important for host defense against infection.

Intracellular TAG/FA cycling plays a central role in β cell insulin secretion, mitochondrial and endoplasmic reticulum (ER) homeostasis. Consistent with transient lipolysis and late DNL, IFNγ upregulated mitochondrial FA oxidation genes, however 24 h exposure to IFNγ led to accumulation of acyl carnitines, suggesting FA overload and limited FA oxidation. IFNγ had minimal impact on glucose oxidation, mitochondrial biogenesis and glucose-stimulated insulin secretion. The IFNγ-induced TAG accumulation at 24 h was insufficient to cause unfolded protein response but increased susceptibility to ER stress induced by interleukin-1β (IL-1β) or tumor necrosis factor α (TNFα). These data suggest that IFNγ enhances DNL for host cell defense in the expense of decreased FA oxidation, and increased risk of cellular stress.

Many cytokines exert their classical biological effects via activation of Janus kinases (JAK) and phosphorylation of Signal Transducer and Activator of Transcription (STAT). IFNγ was shown to regulate lipid metabolism genes in a unique manner compared to type 1 IFN and other inflammatory cytokines, and dependent on signaling through JAK1/2. STAT3 was shown to mediate IFNγ-induced transient lipolysis, however, multiple JAKs/STATs and unphosphorylated STATs could be involved in the constitutive and IFNγ-stimulated expression of genes involved in lipid metabolism in β cells.

In conclusion, this work demonstrates that IFNγ regulates pancreatic β cell lipid metabolism in a dynamic manner that is intimately linked to host defense and cellular function. These findings indicate complex physiological and pathological roles of IFNγ in modulating β cell function, and provide better insight into the mechanism of actions of proinflammatory cytokines in T1D. Targeting lipid metabolism may thus be potential to modulate the effects of proinflammatory cytokines for the prevention and treatment of T1D as well as other inflammatory diseases.

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KEY TO ABBREVIATIONS

ACC	Acetyl CoA carboxylase
ACLY	ATP-citrate lyase
AMPK	AMP kinase
ATGL	Adipose tissue Triglyceride lipase
BSA	Bovine serum albumin
cDNA	Complementary DNA
CE	Cholesterol ester
CPT1	Carnitine palmitoyl transferase
DAG	Diacylglyceride
DAMP	Damage-associated molecular pattern
DGAT	Diacylglycerol acyltransferase
DNL	De novo lipogenesis
ER	Endoplasmic reticulum
ETC	Electron transport chain
FA	Fatty acid
FAO	Fatty acid oxidation
FASN	Fatty acid synthase
FBS	Fetal bovine serum
G0S2	G0/G1 switch protein 2
GSIS	Glucose stimulated insulin secretion
HDL	High-density lipoprotein

IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor
JAK	Janus kinase
LD	Lipid droplet
LPL	Lipoprotein lipase
LXR	Liver X receptor
MAM	Mitochondria-associated (ER) membrane
MAVS	Mitochondria anti-viral signaling
MDA5	Melanoma differentiation-associated gene 5
NAFLD	Non-alcoholic fatty liver disease
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor kB
NOD	Non-obese mice
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate saline buffer
PIC	Polyinosinic:polycytidylic
PLIN	Perilipin
PPAR	Peroxisome proliferator activated receptor

qPCR	Quantitative polymerase chain reaction
RIG-1	Retinoic-acid-inducible gene 1
SREBP	Sterol response element binding protein
STAT	Signal Transducer and Activator of Transcription
TAG	Triacylglyceride
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UBD	Ubiquitin D
UPR	Unfolded protein response
VLDL	Very-low-density lipoprotein

Chapter 1. The impacts of proinflammatory cytokines on cellular fatty acid and cholesterol metabolism - Implications in metabolic diseases and infection

Abstract

Proinflammatory cytokines, e.g. interferons (IFNs), interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα), play a central role in the development of many conditions involving inflammation such as infection, metabolic diseases, and autoimmune diseases. While the inflammatory signaling pathways of these cytokines are well studied, lesser known are their actions on cellular metabolism in target cells and their potential implication. Fatty acid (FA) and cholesterol metabolism have been shown to be altered by cytokines in various tissues. Whether these metabolic changes are physiological or pathological responses remains poorly understood as they are cytokine and cell type dependent. This review first defines the metabolic pathways involving in the regulation of FA, cholesterol and their neutral lipid derivatives metabolism. Next, the effects of proinflammatory cytokines on these pathways in adipocytes, hepatocytes, macrophage-derived foam cells and their implication in metabolic diseases are summarized. An important highlight of this chapter is the emerging role of IFNs on lipid metabolism in immune cells and non-hematopoietic cells and their potential roles in host defense mechanism. Finally, the background on IFN gamma (IFNγ) and its role in pancreatic beta cell function and type 1 diabetes will serve as the rationale for my dissertation in examining the impact of IFNγ on beta cell lipid metabolism.

1.1. Introduction

Cytokines are molecules secreted from immune and non-immune cells during infection or inflammation. They are pivotal for the defense mechanism and maintaining homeostasis, by exerting direct effects against pathogen- or damage- associated molecular patterns (PAMP or DAMP) or stimulating the recruitment, proliferation and activation of immune cells. Unresolved inflammation, however, contributes to tissue damage and dysfunction through the dominance of pro-inflammatory cytokines, e.g. interleukin-1β (IL-1β), tumor necrosis α (TNFα), and interferons (IFNs) versus anti-inflammatory cytokines (IL-10 and IL-13). The collective effects of proinflammatory cytokines on non-immune cells results in enhanced antigen presentation, cellular stress, and programmed cell death. Few studies have demonstrated non-canonical effects of proinflammatory cytokines through changing cellular or systemic lipid metabolism, and how this is associated with disruption of cellular homeostatic function. These studies imply the roles of cytokine-mediated lipid metabolism in the pathogenesis of metabolic diseases including obesity, atherosclerosis and fatty liver disease.

With increasing availability of tools to investigate metabolism at systemic and cellular levels (metabolomics, Seahorse extracellular flux analyzer), the role of lipid metabolism in cellular function is currently an expansive field of research within the last decade. Lipids, especially fatty acids (FAs), cholesterol and their neutral lipids derivatives triacylglyceride (TAG) and cholesterol ester (CE), are reemerging as important species involved in host defense mechanism against infection. Increasing evidence has demonstrated that cytokines can alter FA and cholesterol metabolism of immune cells, and it is correlated with cellular activation. These findings have opened a new era of targeting lipid metabolism for anti-viral or anti-tumor therapy, as well as to reevaluate existing cytokine therapies. Herein I summarize and discuss the impact of

proinflammatory cytokines on intracellular FA and cholesterol metabolism in different cell types and disease-dependent context. Particularly, the role of IFNs on lipid metabolism and host defense response will serve as the rationale to examine the role of IFNγ in pancreatic beta cell, along with its impact on beta cell immune and homeostatic function.

1.2. Proinflammatory cytokines

Proinflammatory cytokines are signaling molecules secreted from immune cells or non-immune cells that stimulate inflammation. These cytokines play a major role in the physiological regulation of host defense in response to PAMP and DAMP. Excessive production of these cytokines, however, causes deleterious effects on the target cells and worsen inflammation. Increased proinflammatory cytokine levels are hallmarks of many chronic inflammatory diseases including metabolic (diabetes, obesity, fatty liver disease), autoimmune, and infectious diseases. The important proinflammatory cytokines discussed in this review are IFN (type 1 and type 2), TNFα, and IL-1β. IL-6 is known to have pleiotropic effects on lipid metabolism and has been reviewed extensively elsewhere [1,2]. Type 1 IFN (IFNα and IFNβ) are produced by most cells during a viral infection [3] and activate anti-viral genes. IFNγ is the only type 2 IFN, and mainly released from CD4+ helper T cells (Th1) and natural killer (NK) cells. Besides anti-viral effect, IFNγ functions as an immunomodulator by recruiting and activating cells of the immune system, especially macrophages [4]. TNFα and IL-1β can be secreted by many cell types, but mostly are produced by monocytes and macrophages [5,6]. The production of proinflammatory cytokines in activated immune cells or non-immune cells are triggered by activation of pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs) and RIG-like receptors (RLRs), leading to transcriptional activation through transcription factors including nuclear factor- κB (NF-κB), Interferon regulatory factor (IRF) and Activator protein -1 (AP-1). Cytokines are released by

exocytosis, and act via an autocrine/paracrine manner by binding to their receptors on target cells. The classical signaling pathway activated by IFNs is Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathways, which upregulate interferon-stimulated genes (ISGs) for anti-viral responses [7]. Both TNFα and IL-1β activate AP-1 and NF-κB-mediated transcription to induce inflammatory responses and mediate diverse effects on cellular function [8,9]. Compared to the well-studied inflammatory signaling of these cytokines (summarized in **Figure 1-1**), their regulation of cellular lipid metabolism and the underlying mechanisms remain under-investigated and are the focus of this review.

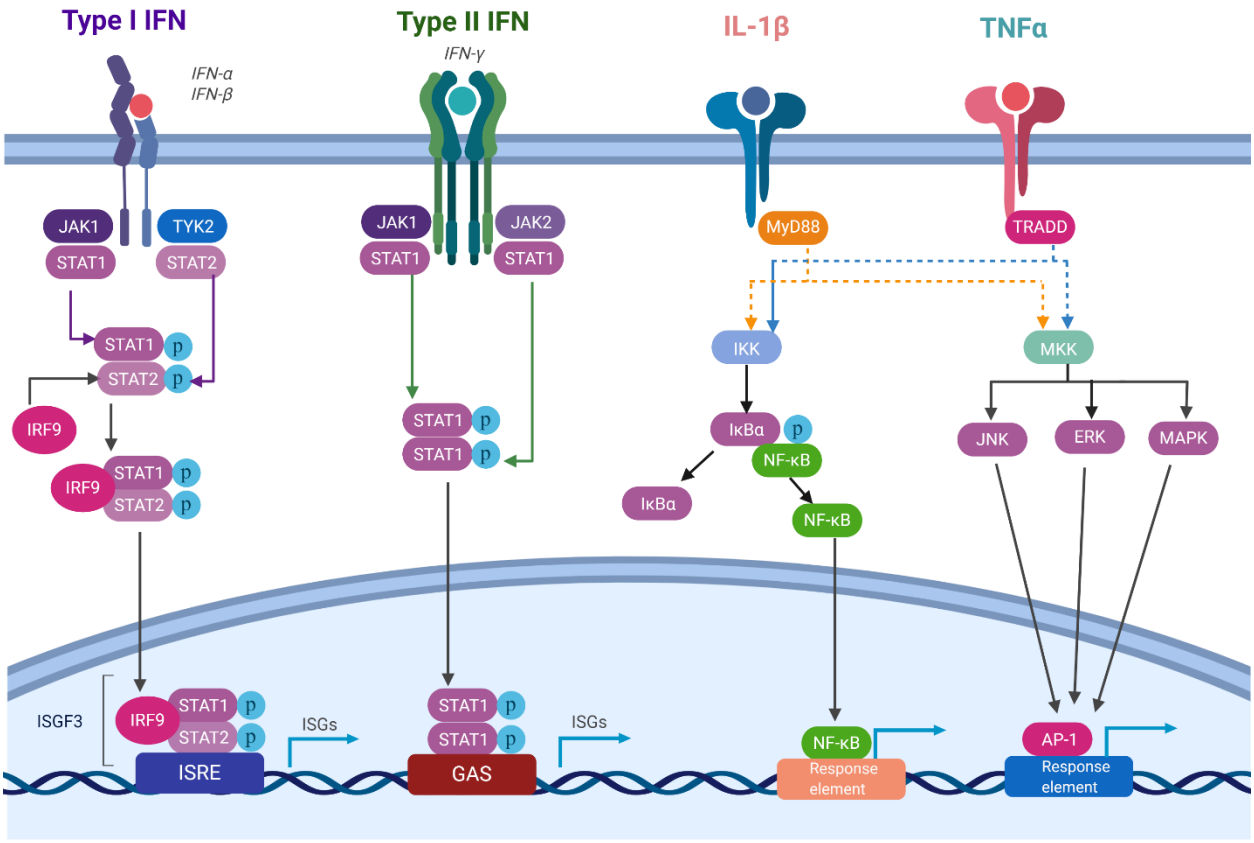


Figure 1-1. Classical signaling pathways activated by proinflammatory cytokines.
IFN type 1 (IFNα, IFNβ) bind to IFNAR, causing receptor conformational change and autophosphorylation of Jak1 and Tyk2 kinases. These kinases in turn phosphorylate STAT protein (mainly at tyrosine residues). Phosphorylated STAT1 and STAT2 form a heterotrimer with the transcription factor IRF9 to form ISGF3 complex, which translocates to the nucleus and binds to the ISRE sequence on gene promoters to initiate transcription of ISGs and regulate anti-viral

Figure 1-1 (cont'd)
function. IFN type 2 (IFNγ), binds to IFNGR and causes activation of JAK1/2 kinases and phosphorylation of STAT1. p-STAT1 homodimers translocate to the nucleus, binds to the GAS sequence and activates transcription of ISGs. IL-1β signaling pathway involves the activation of IL-1R that is bound to the adaptor protein MyD88, leading to a signaling cascade that stimulates MKK and IKK. IKK phosphorylates IκBα, causing the dissociation of IκBα from NF-κB. NF-κB is then free to enter the nucleus and activate expression of genes involved in survival, proliferation and inflammatory response. MKK phosphorylates different kinases of the MAPK family, including ERK, JNK, p38 kinases. These MAPK in turn activates the AP-1 transcription factor, which regulates differentiation, proliferation and apoptosis. TNFα binds to TNFR1 and recruit the transducing molecule TRADD and activates MAPK and NF-κB signaling. JAK: Janus Kinase, TYK: Tyrosine kinase, STAT: Signal Transducer and Activator of Transcription, ISGF3: interferon stimulated gene factor 3, ISRE: Interferon stimulated response element, GAS: gamma activated sequence, ISG: interferon stimulated gene, MyD88: Myeloid differentiation primary response gene 88, MAPK: mitogen activated protein kinase, MKK: MAPK kinase, ERK: extracellular signal-regulated kinases, JNK: Jun N-terminal kinases, AP-1: activator protein-1, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, IκBa: inhibitor of NF-κB, IKK: IκBα kinase, TRADD: TNF receptor-associated death domain

1.3. Fatty acid and cholesterol metabolism pathways and their regulation

In this section, we provide an overview of the synthesis and metabolism of FA, cholesterol and their neutral lipids derivatives, i.e. TAG and CE. These lipid species are the targets of the review because of their central role in metabolic diseases and infection. The interconnected pathways and important enzymes regulating FA and cholesterol metabolism are summarized in **Figure 1-2**.

1.3.1. De novo lipogenesis and cholesterol synthesis

De novo lipogenesis (DNL) is a process of forming FA from other carbon sources, mainly from carbohydrates, in the cytoplasm. DNL can happen at any cell type when carbohydrates are excessive, however, the process is strongly activated in adipose tissue and liver. Pyruvate generated from glycolysis enters the TCA cycle in the mitochondria to form citrate, which is then transported back to the cytosol and converted into acetyl CoA by the enzyme ATP citrate lyase (ACLY). The first committed step of DNL is the carboxylation of acetyl CoA into malonyl CoA, catalyzed by the cytosolic enzyme acetyl CoA carboxylase 1 (ACC1 or ACCα). ACC2 or ACCβ

is localized to mitochondria and catalyzes the formation of malonyl CoA, which inhibits carnitine palmitoyl transferase 1 (CPT1) and FA oxidation in in oxidative tissues [10]. Malonyl CoA is the substrate of fatty acid synthase (FASN), an enzyme complex catalyzing the rate limiting step in DNL to generate palmitic acid. This saturated 16-carbon FA then undergoes further desaturation and elongation by desaturases (SCD) and elongases (ELOV), respectively, to form saturated and unsaturated FAs of different lengths and number of double bonds. Linoleic acid (18:2) and linolenic acid (18:3) are essential FAs which the body cannot synthesize. Their dietary intake therefore is important for the synthesis of essential poly unsaturated fatty acids (PUFAs), such as arachidonic acid (20:4) or docosahexaenoic acid (22:6) [11].

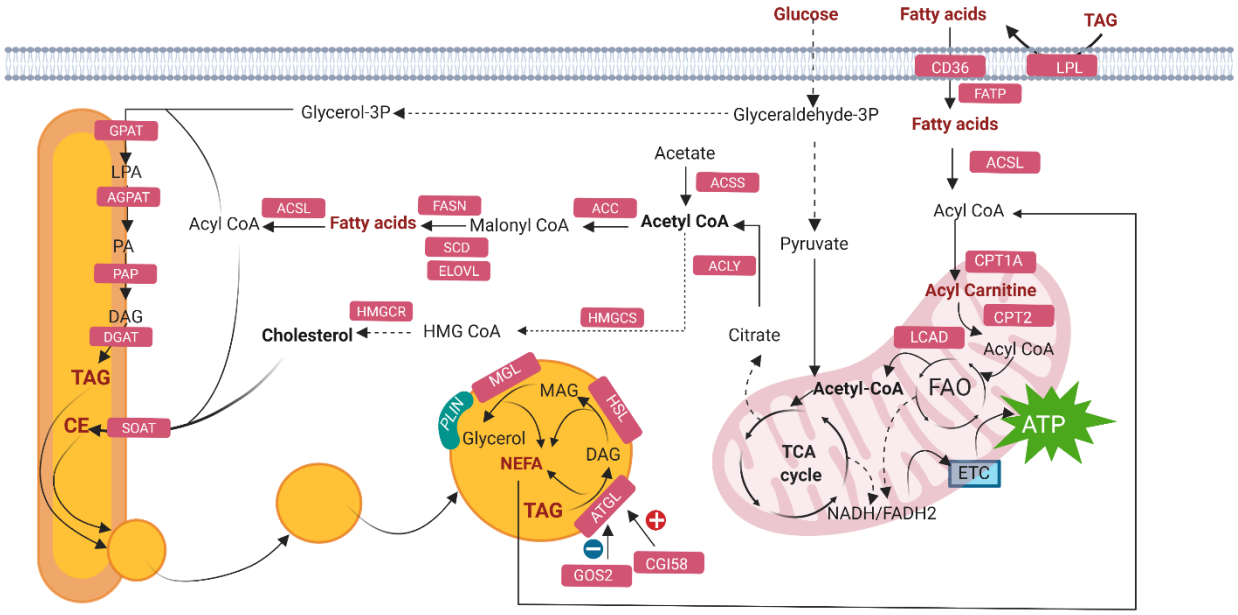


Figure 1-2. Fatty acid and cholesterol metabolism pathways.
The key enzymes are shown in red boxes. One-step reactions are shown as solid arrows, multi-step reactions are shown as dotted arrows. TAG: triacylglyceride, DAG: diacylglyceride, MAG: monoacylglyceride, NEFA: non-esterified fatty acids, FAO: fatty acid oxidation, TCA: tri carboxylic acid, CE: cholesterol ester, LPA: lysophosphatidic acid, PA: phosphatidic acid. Key enzymes/transporters: CD36: fatty acid translocase, LPL: lipoprotein lipase, FATP: fatty acid transport protein, ACSL: long chain acyl CoA synthase, CPT: carnitine palmitoyl transferase, LCAD: long chain acyl dehydrogenase, ATGL: adipose triglyceride lipase, HSL: hormone-sensitive lipase, CGI58: comparative gene identification 58, G0S2: G0/G1 switch gene 2, ACC: acetyl CoA carboxylase, FASN: fatty acid synthase, SCD: stearoyl CoA desaturase, ELOVL: fatty acid elongase, GPAT: Glycerol 3-phosphate acyl transferase, AGPAT: 1-acylglycerol 3-phosphate

Figure 1-2 (cont'd)
acyl transferase, PAP: phosphatidic acid phosphatase, DGAT: diacylglycerol acyltransferase, SOAT: sterol-O-acyltransferase, HMGCS: hydroxymethylglutaryl CoA synthase, HMGCR: hydroxymethylglutaryl CoA reductase.

The pathway for cholesterol synthesis, i.e. the melavonate pathway, also starts from the central precursor cytosolic acetyl CoA. Besides citrate, acetyl CoA can also be formed from acetate by the catalyst of acetyl CoA synthetase short-chain family member 2 (ACSS2). Acetyl CoA is then converted to 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) through the action of acetyl CoA acetyltransferase (ACAT) and HMG CoA synthase (HMGCS). The rate limiting step of this pathway is the formation of melavonate from HMG CoA by the enzyme HMG-CoA reductase (HMGCR). Melavonate is then converted to isoprene, squalene and finally cholesterol, which is used for membrane synthesis or converted into cholesterol ester for storage [12].

1.3.2. Neutral lipid synthesis and formation of lipid droplet

Newly synthesized FAs and cholesterol can be incorporated into glycerolipids, sterol esters, sphingolipid or phospholipid, for storage purpose or formation of membranes. Neutral lipids, i.e. TAG and CE in mammalian cells, are inert lipid species with uncharged groups and therefore unable to be incorporated into plasma membranes but are stored in lipid droplets (LD) as energy reservoir. To form neutral lipids, FA first becomes "activated" by forming acyl CoA using acyl CoA synthase (ACS). Acyl CoA is readily esterified with either glycerol or cholesterol to form TAG or cholesterol ester (CE), respectively. The formation of TAG can begin from two precursors: monoacylglycerol (MAG) in the intestine and glycerol 3 phosphate (G3P) in other tissues (the Kennedy pathway). In the intestine, diacylglyceride (DAG) is created from dietary MAG by the activity of MAG acyltransferase (MGAT). In the Kennedy pathway, G3P esterifies with acyl CoA with the catalyst of G3P O-acyltransferase (GPAT), 1-acyl G3P O-acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP) to form DAG. These two pathways converge at

the final rate limiting step to convert DAG into TAG by diacylglycerol acyltransferase (DGAT) [13]. Unlike TAG synthesis, CE formation is simply controlled by sterol-O-acyl transferase, known as SOAT1/2 (or acyl-CoA cholesterol acyltransferase ACAT) [14].

The synthesis of neutral lipids occurs mainly in the endoplasmic reticulum (ER) membrane where the enzymes are located. After the neutral lipid core of a LD is synthesized between the leaflets of ER bilayer, the so-called lipid lens is budded from the ER to form a nascent LD with a monolayer phospholipid coat derived from ER. Several ER proteins are involved in this budding process, including seipin, lipin and fat induced transmembrane protein (FITM). Once nascent LDs are released from the ER, LD growth or biogenesis can occur by fusion of existing LDs, *in situ* lipid synthesis or exchange of lipid from adjacent ER and mitochondria [15]. LD is considered an organelle with its own unique proteome. Among LD proteins, perilipins (PLIN) on the surface of LDs play significant role in LD growth and mobilization, which will be discussed in the Lipolysis section below.

1.3.3. Lipid uptake and cholesterol transport

The mechanisms for lipid uptake can be different among cell types. In tissue with large storage or oxidative capacity like adipose tissue and skeletal muscle, a large influx of FAs can be obtained from LPL-mediated lipolysis of TAG-rich lipoprotein from chylomicrons or very low-density lipoprotein (VLDL). Uptake of FA liberated from TAG or free FAs in other tissues is critically regulated by the scavenger receptor CD36 (or fatty acid translocase) [16], and other membrane-bound FA transport proteins (FATP) [17]. One across the membrane, FAs are bound to cytoplasmic FA binding proteins (FABP 1-9) and carried to different organelles where they are put into storage or enter metabolic pathways [18]. Macrophages also have the capability to internalized oxidized LDL through class A scavenger receptors (SR-As) and become fat-laden

cells known as “foam” cells [19].

Most cells in the body rely on cholesterol efflux to remove excessive intracellular cholesterol. ATP-binding cassette transporter (ABC) are central in the active form of efflux, carrying cholesterol to the plasma membrane and transport to High density lipoprotein (HDL) or its lipid-poor lipoprotein. HDL cholesterol then can be delivered to liver for cholesterol catabolism and bile synthesis. This form of reverse cholesterol transport is especially important for the removal of cholesterol from foam cells and protection against atherosclerosis [20].

1.3.4. Lipolysis

Neutral lipids undergo hydrolysis to release free FAs for oxidation, membrane synthesis, or signaling. In this review, we focus on TAG lipolysis, a three-step process involving three lipases. Adipose triglyceride lipase (ATGL) catalyzes the first step of breaking down TAG, and hormone sensitive lipase (HSL) is responsible for the hydrolysis of DAG into monoacylglyceride (MAG). In the final step, MAG lipase (MGL) hydrolyzes MAG into glycerol and FFA [21]. A widely recognized stimulant of adipose tissue lipolysis is the hormonal activation of β3-adrenergic receptor by catecholamines, which activates protein kinase A (PKA) and phosphorylates HSL. Increasing evidence, however, argues that ATGL is also the main enzyme that controls lipolysis activity in adipose tissue and many other tissues [21]. ATGL activity is tightly regulated by its coactivator CGI58, co-suppressor G0S2 and perilipins (PLIN) [21]. Under basal conditions, CGI58 is bound to PLIN1 and G0S2 is bound to ATGL. In adipocytes, upon stimulation of lipolysis, phosphorylation of PLIN1 by PKA results in the release of CGI58, allowing CGI58 to bind to ATGL, thereby stimulates its activity [22]. Conversely, endogenous long chain acyl-CoA promoted CGI-58 and PLIN interaction and inhibited lipolysis in skeletal muscle cells [23]. G0S2 expression is downregulated by lipolysis stimulant, releasing ATGL to the LD surface for hydrolysis reaction

[24]. Other PLINs have been shown to participate in the degradation of LDs or protect from lipolysis [25]. PLIN5 is highly expressed in oxidative tissues and has been suggested to play a role in LD-mitochondria FA exchange [26-28].

1.3.5. Fatty acid oxidation and oxidative phosphorylation

FAs released during lipolysis can undergo oxidation to generate ATP to meet cellular energy demands. FA oxidation (FAO) occurs at mitochondria (long chain FAs) or peroxisomes (very long chain FAs), however only mitochondrial FAO is coupled to ATP production. The rate limiting step of mitochondrial FAO is the transport of FA across the mitochondrial outer membrane, achieved by the conversion of long chain acyl CoA into acylcarnitine via carnitine palmitoyltransferase 1 (CPT1). Once acylcarnitine crosses the outer membrane, CPT2 on the inner membrane converts it back into acyl CoA which undergoes beta oxidation in the mitochondria matrix. The initial steps of beta oxidation cycle are catalyzed by dehydrogenases of acyl CoA of different carbon lengths (VLCAD, LCAD, MCAD). Final products of FAO are acetyl CoA molecules, which enter the TCA cycle, and $FADH_2$ and NADH which provide electrons for the oxidation phosphorylation (OXPHOS) inside the electron transport chain (ETC). The ETC is comprised of different enzyme complexes, so-called complexes I-IV, which transfer electrons to oxygen and pump protons across the mitochondrial inner membrane to create an electrochemical proton gradient. This gradient is necessary to activate ATP synthase (complex V) and synthesize ATP for cellular demand [29].

1.3.6. Transcriptional and post-translational regulation of FA and cholesterol metabolism

The expression and activity of the enzymes involved in lipid metabolism pathways are regulated at both transcriptional and post-translational levels. Peroxisome proliferator activated

receptors (PPARs) and Liver X receptors (LXRs), are lipid-sensing nuclear receptors, i.e. they can bind lipid ligands and activate gene transcription [30]. There are three subtypes of PPAR receptor that have distinct expression profiles and functions. PPARγ is mainly expressed in adipose tissue and controls lipid uptake and storage. PPARα is highly expressed in oxidative tissues such as liver, kidney, heart, brown adipose tissue and regulates FA catabolism for ATP production. PPARδ is more ubiquitously expressed and has been shown to regulate lipid metabolism in skeletal muscle and macrophages. Coactivator of PPARγ (PGC1a) plays a critical role in mitochondrial biogenesis, FAO and OXPHOS [31]. LXRs (α and β isoforms) act as cholesterol sensors, and activation of LXR enhances lipogenesis and cholesterol synthesis [32]. Lipid synthesis is also highly regulated by the transcription factors Sterol regulatory element binding proteins (SREBPs). Two major isoforms, SREBP-1 and SREBP-2, are responsible for the expression of genes involved in DNL and cholesterol synthesis, respectively [33]. Besides transcriptional regulation, many important enzymes of lipid metabolism pathways are subjected to post-translational modification for activation or inhibition of activity. AMP kinase (AMPK), an energy sensor whose activity is upregulated during fasting, is a pivotal kinase that controls the phosphorylation of these enzymes. For instance, AMPK inhibits DNL by phosphorylating ACC1, SREBP1 and HMGCR. AMPK also inhibits lipolysis by phosphorylating HSL at the antilipolytic site. Finally, AMPK activates FAO by inhibiting ACC2 function, releasing the inhibitory effect of malonyl CoA on CPT1a activity [34].

1.4. The effects of proinflammatory cytokines on cellular lipid metabolism in metabolically active tissues

Elevated proinflammatory cytokines are hallmarks of obesity, non-alcoholic fatty liver disease (NAFLD) and atherosclerosis. Triggering stimuli, such as infection or tissue damage within adipose tissue, liver and blood vessels, lead to the recruitment and infiltration of immune

cells to the target tissue and amplification of immune response. Cytokines released from immune cells, besides the canonical effects in the inflammatory response, also influence target cell lipid metabolism. The effects can vary drastically among different cytokines and tissues. In this section, we discuss the metabolic effects of proinflammatory cytokines on adipocytes, hepatocytes and macrophage-derived foam cells, which are the most common target of cytokines in metabolic diseases.

1.4.1. Adipocytes

Obesity is characterized by low-grade inflammation with enhanced levels of proinflammatory cytokines, especially TNFα and IL-1β produced by adipocytes and adipose tissue macrophages (ATMs). Proinflammatory cytokines have been shown to inhibit lipid uptake, storage and enhance lipolysis in adipocytes (**Table 1-1**). It is widely accepted LPL, which regulates the FA uptake from TAG-rich chylomicrons or very low-density lipoprotein (VLDL), is the common target of proinflammatory cytokines in adipocytes. TNFα downregulates Lpl mRNA expression and reduces LPL activity in adipocytes cell lines [35], murine [36] and human adipocytes [37,38] and adipose tissue in vivo [39]. IL-1β and IFNs also decrease LPL activity [40,41]. The decrease of LPL activity and/or expression contribute to impaired clearance of TAG from the circulation, leading to hypertriglyceridemia and insulin resistance. TNFα and IL-1β decreased mRNA levels of FA transporters Fatp and Cd36 [42], consistent with the effect on limiting FA uptake in adipose tissues. In addition to reducing the uptake of exogenous FAs, proinflammatory cytokines limit the ability to store lipid in adipocytes by enhancing TAG lipolysis, first shown in 3T3-F442A cell line [36,41]. Ironically, the lipolytic effects were not coordinated with gene expression, as TNFα, IFNα and IFNγ reduced HSL mRNA levels [41]. Recently, the mechanisms have been suggested to involve the downregulation of Pnpla2 and Cgi58 expression and decreased PPARδ binding to G0s2 promoter

by TNFα [43]. A mixture of IFNγ, IL-1β and TNFα increased the expression of NADPH oxidase 3 (NOX3), which was shown to enhance HSL phosphorylation and activate lipolysis [44]. Lipase-independent pro-lipolytic effect was also observed for IFNγ, through inhibiting phosphoenolpyruvate carboxykinase-1 (Pepck-1) expression, thus reducing glycerol synthesis and FA re-esterification [45]. In addition to promoting lipolysis, proinflammatory cytokines also inhibits DNL, by downregulating adipogenic genes, e.g. *Fasn, Acc1, PPARs, Plin1, Srebp1* [41,46,47], resulting in reduced FA synthesis and TAG levels. Collectively, the decrease in DNL, increase in lipolysis and failure to store excessive NEFA in adipocytes caused by proinflammatory cytokines contribute to elevated plasma lipids. This, in turn, leads to FA overload and lipotoxicity in other insulin sensitive organs like liver and skeletal muscle, resulting in increased peripheral insulin resistance [48].

Table 1-1. The effects of cytokines on fatty acid and cholesterol metabolism in adipocytes.

Cytokine	Cell type	Effect	Target genes/proteins	Refs
Lipogenesis				
IFNγ	Human adipocytes	↓TAG	↓PPARδ, *Fasn*, *Plin1*	47
IL-1β	Mouse adipocytes	↓TAG	↓SREBP1, PPARγ, ↓*Fasn*, ↓*Acc*	46
TNFα, IFNα, IFNγ	3T3-F442A adipocytes	↓FA synthesis	↓*Acc* (TNFα), ↓*Fasn* (TNFα, IFNα, IFNβ)	41
IL-1β	3T3-F442A adipocytes	↑ FA synthesis	↑*Acc*	41
Lipolysis				
TNFα, IFNα, IFNβ, IFNγ, IL-1β	3T3-F442A adipocytes	↑		36,40,41
TNFα	3T3-L1 adipocytes	↑	↓*Pnpla2, Cgi-58, G0s2*	43
TNFα+IL-1β+IFNγ	3T3-L1 adipocytes	↑	↑ NOX3 activity, p-HSL	44
IFNγ	Human/mouse adipocytes, 3T3-F442	↑FA release, ↓glycerol synthesis	↓*Pepck-1*, no change in lipases	45
Lipid uptake				
TNFα	3T3-F442A adipocytes, human adipose tissue, 3T3-L1 adipocytes		↓*Lpl* expression and/or activity	35,37,38,40, 41
IL-1β, IFNα, β, γ	3T3-F442A adipocytes		↓LPL activity	40,41
TNFα, IL-1β	Hamster adipose tissues	↓Uptake	↓FATP, ↓CD36	42

1.4.2. Hepatocytes

NAFLD is the most prevalent liver disease characterized by accumulation of fat in hepatic

tissues (so-called steatosis) and is strongly associated with obesity and insulin resistance. Proinflammatory cytokines, especially TNFα and IL-1β, play a central role in the pathogenesis of NAFLD [49]. In contrast with lipolytic effects on adipocytes, proinflammatory cytokines stimulate lipogenesis and inhibit FAO in hepatocytes, leading to accumulation of TAG or hepatic steatosis (**Table 1-2**). TNFα stimulated hepatic lipogenesis in vivo by stimulating FA and sterol synthesis as early as 2 h after administration [50]. Hepatic gene expression revealed that TNFα administration activates genes involved in cholesterol synthesis, FA synthesis and inhibits bile synthesis genes [51] TNFα also stimulated lipogenesis in hepatic cell line HepG2 [52]. There are some studies, however, arguing that TNFα does not have lipogenic effect on primary rat hepatocytes [53], or bovine liver [54]. These contradicting results can arise from different lengths of treatment and whether the effects were assessed *in vitro* or *in vivo*.

Similar to TNFα, IL-1β increases TAG accumulation in Hep G2 [52], and in primary hepatocytes via upregulating *Fasn* [55]. Beside lipogenic effects, TNFα and IL-1β, individually or in combination, decrease FAO *in vivo* [51,56,57], in primary hepatocytes [58] and Hep3B cell line [59,60], through downregulating FAO genes *Ppara*, *Pparg*, *Cpt1a*, *Cpt2* and *Pgc1a*. TNFα and IL-1β also decreased *Fatp* but increased *Cd36* mRNA levels in the liver during sepsis, suggesting decreased FA utilization for FAO but enhancing FA re-esterification into TAG [42]. Increased lipogenesis and failure to oxidize excessive FA can be the main culprits for detrimental effects of these cytokines on liver TAG accumulation.

The role of IFNs to hepatocyte function are more established in viral hepatitis. Due to anti-viral effect, IFNα therapy has been used for treatment of hepatitis C virus infection; yet, it has been shown to alter patients' lipid profile, for instance increasing plasma TAG via lowering LPL activity [61]. The effects of IFNs on hepatocyte lipid metabolism and physiological relevance remains under-

investigated. While IFNα enhanced lipogenesis in HepG2 [52] and mouse liver [62], IFNγ showed no effect [52]. The different impact of two types of IFNs can be attributed to their distinct roles in innate and adaptive immune function and may be responsible for hepatocyte host defense mechanism in viral infection.

Table 1-2. The effects of cytokines on fatty acid and cholesterol metabolism in hepatocytes.

Cytokine	Cell type	Effect	Target genes	Refs
Lipogenesis				
IL-1β, TNFα	Hamster liver	↑ FA synthesis	↑ microsomal ACS activity	57
TNFα	Mouse liver	↑FA synthesis	↑*Fasn*, *Acc-a*	51
TNFα, IL-1β, IFNα	Mouse liver	↑FA synthesis	TNFα increases citrate level	62
TNFα	Bovine liver	No change		54
TNFα, IL-1β, IFNα	HepG2	↑ TAG (IFNγ: no effect)		52
IL-1β	Mouse hepatocytes	↑ TAG	↑*Fasn*	55
TNFα	Mouse liver	↑Cho synthesis ↓Bile synthesis	↑Cholesterogenic genes, ↓cholesterol transport	51
FAO				
TNFα+IL-6 or IL-1β	Rat hepatocytes	↓		58
IL-1β, TNFα	Hamster liver	↓	↓ *Acs1*, mitochondrial ACS activity	57
IL-1β	Mouse liver and hepatocytes	n/d	↓ PPARα, *Cpt1*a expression Inhibit PPARα activity	56
TNFα	Mouse liver	↓	↓ *Cpt1*, *Cact*, *Cpt2*	51
TNFα	Hep3B	↓		59
TNFα, IL-1β	Hep3B	n/d	PPARα,γ, LXR, RXR, CPT1a, SREBP1c	60
Lipid uptake				
TNFα, IL-1β	Hamster liver		↓FATP, ↑CD36	42

1.4.3. Macrophage-derived foam cells

Macrophage-derived foam cells play a central role in atherosclerosis and contribute to the development of cardiovascular disease. They are anti-inflammatory (M2) macrophages loaded with neutral lipids, particularly CE in those residing at atherosclerotic plaques. Their formation occurs as monocytes are recruited to atherosclerotic site within blood vessel walls and start to differentiate into macrophages. Macrophage-derived foam cells have scavenger receptors on their surface that can internalize circulating low density lipoprotein (LDL)-bound cholesterol, especially oxidized LDL (oxLDL) from the bloodstream [63]. Once inside the cell, cholesterol esters from LDL

are hydrolyzed to free cholesterol, which is transported to the ER for re-esterification into CE for storage. Foam cells can also export free cholesterol to high density lipoprotein (HDL) to transport back to the liver for catabolism. Generally, cytokines have been shown to increase lipid accumulation in macrophages-derived foam cells by multiple mechanisms (**Table 1-3**). TNFα, IL-1β increased ER ACS activity while decreasing mitochondrial one, suggesting enhanced FA esterification for storage. In addition, they decrease FA efflux, and collectively caused TAG and cholesterol accumulation in human macrophages and human monocytic cell line-derived foam cells [64]. TNFα and IL-1β also downregulated ABC transporter mRNA levels in murine macrophage cell line, indicating reduced cholesterol efflux [65].

Within the IFN family, the effects of type 1 and type 2 IFN on cholesterol metabolism in macrophage-derived foams cells are heterogenous. IFNγ increased CE synthesis by upregulating SOAT levels and reduced cholesterol efflux in murine foam cells [66]. In addition, IFNγ diminished mRNA and protein levels of cholesterol 27-OH hydroxylase, which enhanced cholesterol accumulation and support foam cells formation from LDL-treated THP-1 macrophage cell line [67]. In contrast to these studies, IFNγ downregulated the scavenger receptor expression and limited LDL uptake, thus inhibiting foam cell formation from human monocytes-derived macrophages [68]. Type 1 IFNs appear to promote atherosclerosis by enhancing macrophage adhesion and recruitment of leukocyte to plaques [69]. Mechanistically, IFNα and IFNβ were shown to upregulate scavenger receptor A [70] and downregulate ABCA1 expression, suggesting increased uptake and decreased efflux in foam cells *in vitro* and *ex vivo* [71]. IFNβ administration also increased lipid accumulation in peritoneal macrophages of atherosclerotic mouse model ($LDLR^{-/-}$ mice fed with high fat diet) [71].

It is noteworthy that the macrophages used in these studies were differentiated *in vitro* from

different sources. Studies have shown that bone marrow-derived macrophages (BMDM), peritoneal macrophages and peripheral blood mononuclear cells-derived macrophages (mDM) can polarize differently towards the inflammatory (M1) or anti-inflammatory state (M2) [72], especially in response to oxidize LDL [73]. More *in vivo* studies in atherosclerotic animal models are necessary to examine the effects of cytokines for better understanding of their metabolic impacts on macrophage phagocytic function and implication in the cardiovascular disease.

Table 1-3. The effects of cytokines on fatty acid and cholesterol metabolism in macrophage-derived foam cells.

Cytokine	Cell type	Effect	Target genes	Refs
Lipogenesis				
IL-1β, TNFα	Human mDM, THP-1 human macrophage	↑ TAG	↑ER ↓ Mito ACS activity (esterification), ↓ FA efflux	64
Lipid uptake				
IFNγ	Human mDM	↓ LDL uptake	↓ scavenger receptor	68
IFNβ	Mouse BMDM and human mDM	↑ LDL uptake	↑ scavenger receptor A (Sc-RA)	71
IFNα	Human mDM, THP-1	↑ox- LDL uptake	↑ Sc-RA	70
Cholesterol metabolism and efflux				
IL-1β, TNFα	J774 murine macrophage		↓ *Abca1*, *Abcg1*	65
IL-1β, TNFα	Human mDM, THP-1	↑ Cholesterol accumulation, ↓ Efflux		64
IFNγ	Mouse peritoneal macrophages	↑ CE synthesis	↑*Soat*	66
IFNγ	THP-1	↑	↓25-OH cholesterol hydroxylase levels	67
IFNβ	Mouse BMDM and human mDM, in vivo mouse peritoneal macrophages	↓ efflux, ↑ lipid accumulation (in vivo)	↓ ABCA1	71

1.4.4. Alteration of intracellular lipid metabolism by cytokines and its link to host defense

Studies in metabolic tissues have demonstrated that proinflammatory cytokines generally impair lipid storage in adipocytes and lipid oxidation in hepatocytes, leading to disruption of metabolic homeostasis and contribute to the pathogenesis of metabolic diseases. Nonetheless, the fact that certain cytokines stimulate oxLDL uptake by macrophages argues that cytokine-mediated metabolic effects can be a physiological response to protect from harmful environmental insults.

In line with these observations, growing evidence has demonstrated that immune cells, especially phagocytic cells, and non-hematopoietic cells undergo lipid reprogramming after exposure to cytokines to mount a host cell defense response (**Table 1-4**).

Among lipid metabolism pathways, DNL and cholesterol synthesis have become the center of attention as they play many roles in inflammation and host defense. Host cell *de novo* FA synthesis is crucial for infection and survival of many pathogens, notably viruses [74] and *Mycobacterium tuberculosis* (*Mtb*) [75]. Although the role of DNL in infected cells is unknown, studies have indicated its important impact on immune cell function and its regulation by cytokines. TNFα, IFNγ or their combination upregulated ACLY gene and protein expression in human monocyte-derived macrophages and macrophage cell line U937, and this was required for ROS production and macrophage activation [76]. They also upregulated mitochondrial citrate carrier *Slc25a1* expression and induced level of cytosolic citrate and acetyl CoA, which are necessary for TNFα and IFNγ-mediated NO and prostaglandin production in U937 cells [77]. While the mechanism whereby DNL and its metabolites regulate macrophage production of inflammatory signals is not yet reported, NF-κB signaling might be involved as it was shown to be associated with FA-induced NO production in macrophages [78]. The TCA cycle also appears to be targeted by cytokines for host defense. TNFα, IFNγ and recently IFNβ have been shown to induce Immunosuppressive gene 1 (*Irg1)* expression and increases the conversion of aconitate into itaconate, a metabolite produced in large quantities in activated macrophages with potent anti-inflammatory effects [79-81].

Cholesterol is important for membrane synthesis and therefore is targeted by viruses to facilitate host cell evasion and viral replication. As DNL and cholesterol synthesis occur from the same central precursor acetyl CoA, it is speculated that these pathways may share common roles in host defense. Indeed, aceto-acetyl CoA, an intermediate metabolite of *de novo* FA synthesis, is

required for cholesterol synthesis, which is important for TLR4 to enter lipid rafts and become activated [82]. IFNs have been shown to regulate both FA and cholesterol synthesis in immune cells and this is directly linked to immune cell activation. IFNγ or IFNβ, but not TNFα, IL-1β or IL-6 downregulated cholesterol synthesis genes in BDDMs and fibroblasts. Inhibition of mevalonate-isoprenoid branch by statin or IFNβ in macrophages limited viral growth *in vitro* and *in vivo* [83]. To support this study, IFNβ was shown to inhibit both *de novo* FA and cholesterol synthesis in BMDMs. In return, deletion of *Scap*, a transcription factor controlling the cholesterol synthesis, induced type 1 IFN secretion and protected from viral infection [84]. Besides IFNs, other cytokines can participate in anti-viral mechanism through changing cholesterol metabolism. IL-1β, TNFα, Zika virus, and TLR stimulation promote the conversion of cholesterol to 25-hydroxycholesterol (25-HC), an anti-viral metabolite, in human macrophage cell line THP-1 [85]. Interestingly, while virus-induced 25-HC formation was independent of type 1 IFN, the cytokine-mediated effect was dependent upon STAT1 signaling, suggesting a cross talk among cytokines and downstream STAT signaling pathway in regulation of cholesterol metabolism.

Lipid droplet, traditionally seen as a pure storage compartment of excessive FA and cholesterol, has emerged to be a functional organelle that interact directly with pathogens. The central theory argues that viruses, bacteria and parasites hijack host cell LDs to support their survival and infection by obtaining FAs for energy, synthesis or using LDs as platform for replication [86]. Growing evidence has suggested that LDs play active role in host defense mechanism. Viperin, an interferon-stimulated protein with direct anti-viral function, localizes on LD surface [87]. Limiting LD density in HeLa cells decreased type 1 and type 3 IFN production and ISGs expression, suggesting impaired anti-viral function [88]. The effect of cytokines on LD biogenesis have not been thoroughly studied and mostly focused on macrophages. In *Mtb*- infected

monocytes, there was a marked increase in the number of LDs, whose formation was dependent upon IFNγ and exogenous FA uptake through CD36. The accumulation of these LDs was demonstrated to be necessary to prevent *Mtb* utilization of intracellular FAs [89]. This study supports previous findings on IFNγ-induced LD formation in human monocytes [90] and Rickettsia infected fibroblasts [91] indicating that IFNγ can enhance storage of FAs into TAG and LDs in response to infection. Collectively, these studies have demonstrated a role of IFNγ in the regulation of LD biogenesis in immune and non-immune cells, and this change might be crucial for anti-pathogen function. In addition to IFNγ, TNF signaling was also linked to TAG accumulation in macrophage-derived foam cells in tuberculous lung granulomas [92], although the mechanism and the physiological relevance is not well understood. Ironically, TNFα was originally shown to downregulated *Lpl* expression in a macrophage cell line [93]. The effects of proinflammatory cytokines and interferons on LD biogenesis, especially in infected cells, and their importance in host defense remains unexplored.

Type 1 IFNs have been specifically shown to target OXPHOS of immune and non-immune cells. OXPHOS is a more efficient process to generate ATP and usually dominant in resting immune cells, while glycolysis is robustly enhanced in activated immune cells. IFNα activated conventional DCs in response to poly IC by switching from OXPHOS to glycolysis, a process dependent of hypoxia-inducible factor a1a (*Hif1a*) [94]. In plasmacytoid DCs, IFNα enhanced FAS, FAO and OXPHOS through PPARα [95]. The different actions of IFNα suggest that IFN-mediated effects on OXPHOS and mitochondrial capacity are cell-specific, and likely regulate different cellular responses. Importantly, IFNα was shown enhanced OXPHOS in memory CD8+ T cells and keratinocytes [95]. The change in keratinocytes OXPHOS was necessary for positive feedback on type 1 IFN production and mounting an anti-viral response. These findings have established a

novel link between IFN-induced changes in host cell lipid metabolism to the immune function in non-hematopoietic cells.

Table 1-4. The effects of cytokines on fatty acid and cholesterol metabolism in immune cells and infected host cells.

Cytokine	Cell type	Effect	Target genes	Refs
Lipogenesis and Cholesterol metabolism				
TNFα, IFNγ	Human macrophages, U937	Not discussed	↑*Acly*, ↑*Slc25a*	76,77
IFNγ	TB infected mouse BMDM	↑ LD	CD36 is required	89
TNFα, IFNγ	Mouse BMDM		↑IRG1	81
IFNβ	Mouse BMDM	↑Itaconate	↑ *Irg1*	79,80
IFNβ, IFNγ	Mouse BMDM	↓Cho synthesis	↓*Srebp2, Hmgcs1, Hmgcr, Idl1, Sqle*	83
IFNβ	Mouse BMDM	↓Cho synthesis, ↓FA 18:0, 16:0	↓*Sqle*, ↓ FASN	84
TNFα, IL-1β	Human mDM, THP-1	↑25-Hydroxy -cholesterol (25HC)		85
Lipid uptake				
IFNγ	Human monocytes	Incorporation of exogenous FA		90
TNFα	Murine RAW 264.7 macrophages	N/A	↓*Lpl*	93
OXPHOS				
IFNα	Mouse dendritic cells	↓ OXPHOS		94
IFNα	Mouse plasmacytoid dendritic cells, PDV mouse keratinocytes	↑ FAO, OXPHOS		95

Given increasing evidence linking IFN-mediated lipid metabolism to host defense function, it is necessary to re-examine the metabolic effects of proinflammatory cytokines in adipocytes, hepatocytes and other non-immune cells and whether they contribute to immune response. It was suggested that adipocyte-derived lipolysis could contribute to innate immune response via multiple roles. First, lipolysis-derived FA can be important for the formation of VLDL and encapsulation of pathogen, e.g. LPS. Second, FA could also support mitochondrial FAO, a process important for M2 macrophage and regulatory T cell activation [96]. Nutrients exchange has been demonstrated in the tumor microenvironment, where tumor metabolism reciprocally alters immune cell function [97]. Finally, FAs can serve as a ligand for TLR4 and potentiate the inflammatory state of both adipocytes and macrophages [98]. Conversely, a recent

study argued that adipocyte lipolysis does not alter ATM function, but merely enhance their capacity to take up FA and put into storage [99]. Understanding the physiological role of cytokine-mediated effects on cellular lipid metabolism of non-immune cells, i.e. whether play an active role in immune function, is extremely crucial for development of cytokine-targeting therapies.

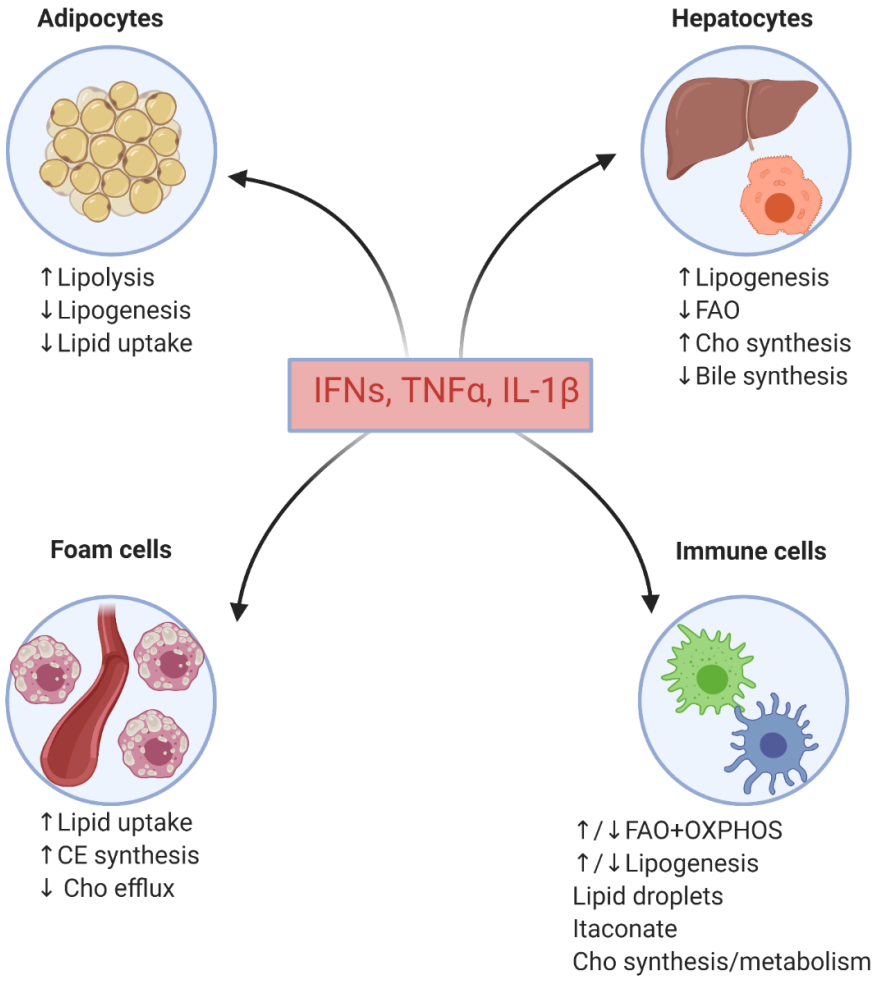


Figure 1-3. Summary of the effects of proinflammatory cytokines on cellular metabolism. In adipocytes, IFNs, TNFα and IL-1β generally enhance TAG lipolysis, decrease lipogenesis and uptake of exogenous FA, causing elevated plasma free FAs and insulin resistance. In contrast, proinflammatory cytokines cause TAG accumulation in hepatocytes by increasing lipogenesis and decreasing FAO. They can also increase cholesterol synthesis and decrease bile synthesis, resulting in cholesterol accumulation in the liver. In macrophage-derived foam cells, cytokines enhance uptake of lipid, especially oxidized LDL and increase cholesterol ester synthesis and storage. At the same time, cytokines also inhibit reverse cholesterol efflux, causing accumulation of

Figure 1-3 (cont'd)
cholesterol in the macrophages. The effects of cytokines on immune cell lipid metabolism are more variable and are linked to host defense function. IFN type 1 limit lipogenesis and cholesterol synthesis but have differential effects on FAO and OXPHOS depending on immune cell types. IFNγ and TNFα enhanced lipogenesis and lipid droplet formation in macrophages. Finally, certain cytokines can affect TCA cycle metabolite and cholesterol metabolism.

1.5. The roles of IFNγ in pancreatic beta cell dysfunction and development of type 1 diabetes

1.5.1. Type 1 diabetes and its link to viral infection

Type 1 diabetes (T1D), also known as juvenile diabetes and insulin-dependent diabetes mellitus, is a chronic autoimmune disease that affects millions of children and young adults around the world [100]. It is characterized by the self-recognition of pancreatic β cell antigens, leading to T cell-mediated β cell destruction and loss of insulin secretion. Lack of insulin causes loss of glucose control, leading to hyperglycemia, increased thirst (polydipsia), increased urination (polyuria) and weight loss. There is currently no cure for T1D; patients with the disease are dependent on insulin injection to maintain blood glucose levels.

It is believed that islets autoantigen such as insulin, glutamic acid decarboxylase 65 (GAD65) and insulinoma antigen-2 (IA-2) are presented by antigen presenting cells to naïve T cells at the pancreatic lymph nodes to generate autoreactive T helper cells (CD4+ T cells) [101]. Effector T cells infiltrate islets and secret cytokines that stimulate the recruitment and activation of other immune cells, including macrophages and cytotoxic T cells (CD8+), leading to cell-mediated cytotoxicity and β cell destruction. Proinflammatory cytokines IFNγ, IL-1β, and TNFα are secreted by immune cells, specially CD4+, CD8+ T cells and macrophages, and are well known to have direct detrimental effects on β cells, causing cellular dysfunction and apoptosis [102].

T1D is a disease involving both genetic risks and environmental factors. A general hypothesis is that in genetically susceptible individuals, exposure to certain environmental factors

trigger islet autoimmunity. Viral infections have been suggested to have a tight relation to the development of T1D. Enteroviruses, especially coxsackievirus B (CVB), have been implicated in the initiation or progression of islet autoimmunity [103]. Although there are some rare reports of viral presence in human islets [104,105], most studies suggest that persistent or recurrent viral infection breaks immune tolerance and increases beta cell recognition by autoreactive T cells [106,107]. The activation of innate viral receptors and STAT1 in islets of individuals with T1D support the link between viral infection, interferon signaling, and islet autoimmunity [107,108]. For example, the increase in plasma and islets IFNα levels correlated with CVB infection patients with T1D [109]. Gain-of-function mutation or deletion of the *IFIH1* gene, which encodes for the viral recognition receptor melanoma differentiation-associated protein 5 (MDA5), have been shown to protect from T1D in human and rodent models [110,111]. These studies demonstrate a pivotal role of anti-viral interferons and host defense signaling of the pancreatic islets in the initiation and progression of T1D.

1.5.2. Multifaceted role of IFNγ on β cell function

IFNγ is a Th1 cell cytokine that plays a central role in anti-viral response, activation of immune cells, and stimulation of immune responses. IFNγ has been implicated in β cell destruction and T1D pathogenesis. Overexpression of IFNγ in β cells caused loss of islet tolerance through enhancing lymphocytes infiltration and lymphocyte-mediated islet destruction [112]. IFNγ, but not IFNα, caused upregulation of MHC class I [113,114], thus increased β cell recognition by CD8+ T cell, and decreased insulin release and content in β cell lines [115]. The effects of IFNγ on insulin synthesis was suggested to be mediated through the decrease of β cell transcription factor PDX-1 nuclear localization [116].

IFNγ is frequently used in combination with IL-1β and/or TNFα to mimic the condition of

proinflammatory cytokines in the islet environment, and IFNγ has been shown to play central role in cytokine-induced β cell dysfunction. IFNGR knockout islets are resistant to IFNγ+IL1β -mediated NO production and impaired insulin secretion [117]. IFNγ-induced STAT1 activation is shown to be responsible for cytokine-induced apoptosis and downregulation of genes involved in β cell development (Ins, Pdx-1, MafA, Glut2) [118]. IFNγ deficient mice did not develop T1D even though β cells express antigens specific for CD8+ T cell-mediated cytotoxicity [119].

Although many reports suggest that IFNγ causes β cell dysfunction, there are other lines of evidence suggesting that IFNγ is dispensable for T1D. Transgenic non obese diabetes (NOD) mice with β cell IFNGR deletion developed T1D spontaneously at the same rate as WT NOD mice [114]. Whole body knockout of IFNGR mice also showed no difference in the rate of development of T1D induced by streptozocin [120]. IFNγ was shown to promote the expression of program-death ligand 1 (Pd1) in islets, whose expression correlates with insulitis in T1D, suggesting IFNγ limits autoreactive T cell function [121]. Increasing dose of IFNγ administration to NOD mice inhibits the development of T1D by limiting effector T cells [122]. Low populations of CD4+ and CD8+ T cells and low production of IFNγ were observed in patients at onset of T1D compared to non-diabetic high-risk individuals [123]. This evidence demonstrates that IFNγ is a cytokine with multifaceted function in both innate and adaptive immunity and its role on β cell dysfunction requires more investigation.

1.5.3. Beta cell intracellular lipid metabolism regulates cellular function

The main function of β cells is to synthesize and secrete insulin for the regulation of plasma glucose levels. Insulin secretion is tightly coupled to metabolism. The classical mechanism starts with glucose entering the β cell through GLUT2 and undergo glycolysis to generate pyruvate, which enters the TCA cycle and produce ATP. The rise in the ATP/ADP ratio triggers the closure

of ATP-dependent potassium channel, leading to depolarization of the plasma membrane. Upon reaching the threshold, voltage-gated calcium channels open, allowing Ca^{2+} influx into β cells and stimulation of exocytosis of insulin-contained vesicles. The release of insulin follows a dynamic pattern: the triggering phase with a sharp increase in insulin levels followed by a prolonged amplifying phase. Exogenous and intracellular FAs have been shown to potentiate insulin release. The mechanisms include the formation of long chain acyl CoA as signaling molecules. Specifically, the anaplerotic metabolism of glucose increases the production of malonyl CoA, which inhibits CPT1a and mitochondrial FAO, thus increasing levels of long chain acyl CoAs [124,125]. These acyl CoAs have been shown to stimulate insulin secretion in a K_{ATP}-independent manner [126]. To support these studies, lowering malonyl CoA by overexpressing malonyl CoA decarboxylase impairs glucose plus FA-induced insulin release [127]. Inhibition or stimulation of CPT1a and FAO enhance or inhibit GSIS, respectively [125,128,129]. In addition to the formation of malonyl CoA from the anaplerotic glucose metabolism or exogenous FAs, the intracellular TAG/FFA cycle is also critical for FA partitioning and generation of FA signaling molecule for insulin secretion. Inhibition of TAG lipolysis by orlistat markedly reduced GSIS in rat islets [130]. B cell-specific knockout of HSL or ATGL exhibit impaired insulin release [131,132]. These studies strongly suggest that FFAs derived from lipolysis are important for insulin secretion.

It should be noted that chronic exposure to saturated NEFAs such as palmitic acid (16:0), however, impairs insulin secretion through lipotoxicity. Saturated long chain FAs are well known to enhance β cell ER stress, mitochondrial stress, and cell death, leading to loss of insulin release [133,134]. On the other hand, unsaturated FAs exert a much milder effect on β cells, and even protective against cytokine-induced apoptosis [135,136]. Unsaturated, but not saturated FAs are able to be put into storage in LDs [137], indicating an important role of LDs in protecting β cells from

lipotoxicity. Accordingly, many LD proteins like PLINs have been shown to play active role in β cell homeostasis [138,139]. Whereas the role of intracellular lipid to β cell stress and insulin secretion has gained a lot of attention, there is a current lack of understanding on whether it regulates β cell host defense function, particularly in inflammatory conditions of diabetes.

1.6. Goal of dissertation

IFNs have been recognized to play a crucial role in the regulation of lipid metabolism in immune cells that is directly linked to cellular activation and host defense. T1D is an autoimmune disease with a strong association with virus-induced immunity, and proinflammatory cytokines are known to have detrimental effect on of β cell loss and dysfunction. IFNγ is a proinflammatory cytokine with innate anti-viral function, and its multifaceted role in β cell function and the development of T1D is inadequately understood. My dissertation therefore will investigate the impacts of IFNγ on pancreatic β cell following three specific aims:

Aim 1. Characterize the effect of IFNγ on intracellular lipid metabolism and its association with host defense

Hypothesis: IFNγ alters pancreatic β cell intracellular FA and cholesterol metabolism, which plays a role in anti-viral response.

Methods: Pancreatic β cells INS-1 or primary islets were treated *in vitro* with IFNγ. Non targeted lipidomics and quantitative PCR were performed to address time-dependent change in lipid compositions and lipid metabolism gene expression in β cells. The effects of lipid metabolism on anti-viral response will be examined via gene expression of anti-viral gene following blockade of lipid metabolism pathways.

Aim 2. Examine the effect of IFNγ on mitochondrial and ER function

Hypothesis: IFNγ-mediated lipid metabolism affects mitochondrial and ER function that is

associated with secretory function and ER stress.

Methods: Mitochondrial function of IFNγ-treated INS-1 cells will be addressed via cellular respiration to different substrates, protein and mRNA markers of mitochondrial oxidation and biogenesis. IFNγ-mediated effect on insulin secretion will be determined. Finally, the effects of IFNγ by itself or with other proinflammatory cytokines on ER function will be examined by gene expression of ER stress.

Aim 3. Identify signaling pathways that regulate IFNγ-mediated transcriptional effect

Hypothesis: IFNγ signals through classical JAK/STAT pathway to regulate lipid metabolism.

Methods: Pharmacological inhibitor and small-interfering RNA against different components of JAK/STAT pathways will be used to examine the signaling pathway involved in IFNγ-mediated transcriptional regulation of lipid metabolism.

Chapter 2. The impact of interferon gamma on pancreatic beta cell lipid metabolism and its association with host defense mechanism

Abstract

Viral infection has been implicated in the pathogenesis of type 1 diabetes; however, the effects of viral infection and anti-viral cytokines, i.e. interferons on pancreatic islet and β cell metabolism have not been characterized. In this study, we showed that administration of viral mimetic polyinosinic: polycytidylic (PIC) to LEW.1WR1 rats results in a significant increase of triacylglyceride (TAG) levels in pancreatic islets, preceding the onset of insulitis and type 1 diabetes. Elevated levels of interferon gamma (IFNγ) -induced genes were observed in these islets, suggesting a role of IFNγ in regulating islets lipid metabolism. Next, the effects of IFNγ on β cell lipid metabolism was assessed *in vitro* in INS-1 cells. Treatment of INS-1 cells with IFNγ for 6 to 24 h led to a dynamic change in TAG levels and lipid droplets (LD) numbers: a decrease at 6 h and an increase at 24 h. Gene expression results show that IFNγ regulates TAG lipolysis and LD mobilization genes, e.g. *G0s2*, *Pnpla2*, *Plin* in a dynamic manner. The late accumulation of TAG in INS-1 cells was associated with elevated non-esterified fatty acids levels and increased expression of genes regulating *de novo* lipogenesis (DNL). We proposed that IFNγ-induced DNL and TAG accumulation at 24 h contributes to IFNγ-mediated effects in host defense. Pretreatment with IFNγ robustly enhanced PIC-induced anti-viral gene expressions, and this potentiating effect of IFNγ was abolished by pharmacological inhibitors of DNL. Our studies demonstrated a non-canonical effect of IFNγ in regulation of pancreatic β cell lipid metabolism that is intimately linked with a host defense mechanism.

2.1. Introduction

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-producing pancreatic β cells leading to insulin deficiency and loss of glycemic control. Although it is recognized that adaptive and innate immune cells infiltrate pancreatic islets and release inflammatory cytokines including IFNγ, IL-1β and TNFα, the effect of these signaling molecules on β cell metabolism and stress as it relates to early disease progression is not well understood. IFNγ is a type 2 IFN secreted by T cells, antigen-presenting cells, and natural killer cells, and possesses both anti-viral and immune-modulatory functions [4]. In β cells, IFNγ upregulates major histocompatibility complex class I expression [113] and increases the susceptibility to apoptosis induced by IL-1β or TNFα [102,140] or viral mimetic [141]. IFNγ also promotes recruitment of cytotoxic T cells to islets [142]. Other studies, however, have shown that IFNγ can activate protective signaling pathways [120] and can limit autoreactive T cell function [121], emphasizing the lack of understanding about the pleiotropic effects of IFNγ in the pathogenesis of T1D.

Inflammatory cytokines have been shown to alter lipid metabolism in many cellular systems (reviewed in Chapter 1). For example, IFNγ, IL-1β and TNFα stimulate lipolysis in adipocytes [143], yet enhance lipid accumulation in hepatocytes and macrophage-derived foam cells [51,64]. In relationship to pathogen infection, type 1 IFNs produce metabolic effects associated with host defense in both immune and non-hematopoietic cells. For instance, IFNα increases fatty acid oxidation (FAO) and oxidative phosphorylation in plasmacytoid dendritic (pDC) cells and keratinocytes, and this is necessary for full pDC cell activation and anti-viral response in keratinocytes [95]. In macrophages, IFNβ was shown to limit cholesterol synthesis and increase import of cholesterol and long chain fatty acids (FA), while blocking cholesterol synthesis spontaneously activated a type 1 IFN response [84]. Host cell signaling triggered by pathogen-

associated molecular patterns (PAMP) also alter metabolism of lipids, including triacylglyceride (TAG), lipid droplets (LD), FA and cholesterol, to enhance host defense [82,144,145]. Whether inflammatory cytokines alter lipid metabolism in pancreatic islets and β cells and its impact in host cell defense function and progression of T1D has not been reported.

To explore the relationship between β cell autoinflammation and changes in the islet lipidome *in vivo*, we employed the inducible T1D LEW.1WR1 rat model. Injection of polyinosinic:polycytidylic (PIC) induces marked insulitis and T1D after 11 to 34 days in both male and female LEW.1WR1 rats [146]. Susceptibility of LEW.1WR1 rats to PIC- and viral-induced T1D lies, in part, within the major susceptibility locus *Iddm37*, which is adjacent to the major histocompatibility complex (MHC), that contains the IFNγ-regulated *Ubd* (ubiquitin D) gene [147,148] and is dependent on type 1 IFN signaling [149]. Primary rat islets and the rat β cell line INS-1 were then employed to investigate the impact of IFNγ on β cell lipid metabolism and its relationship to host cell anti-viral response.

2.2. Materials and methods

Animals and islet isolation. All animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University. LEW.1WR1 rats 27 to 31 days of age (Biomere, MA) were housed under a controlled temperature of 25°C and a 12-h light cycle, with *ad libitum* access to water and standard chow (Envigo, UK) and allowed to acclimate for 1 week. Rats were divided randomly into 2 groups and received intraperitoneal (i.p.) injection of either PBS (control) or PIC (1 μg/g body weight; Invivogen, CA) every other day for up to 14 consecutive days. For immunohistochemical analysis, LEW.1WR1 rats were treated with PIC for 6, 8 or 12 days, after which rats were fasted (6 h), anesthetized with isoflurane, and exsanguinated. Pancreata were removed, fixed in 10% formalin, embedded with paraffin, sectioned (5 μm),

deparaffinated, stained with hematoxylin-eosin and immunohistochemically stained using rabbit polyclonal anti-CD3 (Abcam, MA) and mouse monoclonal anti-CD68 antibodies (MilliporeSigma, MO). Twelve islets per section were graded by two unbiased evaluators using a scale of 0 to 4+ as follows: 0, no infiltrating CD3+ or CD68+ cells into any islets when compared to controls; 1+, small numbers of CD3+ or CD68+ cells restricted primarily to the islet periphery with preservation of islet architecture; 2+, small numbers of islet infiltrating CD3+ or CD68+ cells with preservation of islet architecture; 3+, large numbers of islet infiltrating CD3+ or CD68+ cells with distortion islet architecture; and 4+, complete loss of islets. For lipidomic analysis of isolated islets, rats were treated with PIC or PBS for 4 days, and forty-eight hours after the last PIC injection (4D+48h pi), islets were dissociated from pancreatic tissue using Clzyme RI (VitaCyte LLC, IN), purified by Histopaque (Sigma, MO) and centrifugation, handpicked, and lipids extracted (see below). For mRNA expression, rats were treated with PIC or PBS for 4 days or 6 days, and 16 h or 48 h after the last injection (4D+48h pi, 6D+16h pi, 6D+48h pi), islets were isolated and RNA extracted (see below).

Cell culture. INS-1 cells (received from C. Wollheim, Geneva, Switzerland) were maintained in RPMI 1640 media containing 10% FBS, 1 mM pyruvate, 10 mM HEPES, 50 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (INS-1 media). For IFNγ experiments, cells were plated at a density of 0.2 x 10^6 cells/cm^2 and cultured in INS-1 media for 24 h and then in INS-1 media containing 10% heat-inactivated FBS (hi-FBS) for 24 h. Cells were then cultured for indicated lengths of time with 50 ng/ml rat recombinant IFNγ (R&D systems, MN) or vehicle (0.00005% BSA/PBS) in INS-1 media containing 10% hi-FBS and then harvested for mRNA, protein, or lipidome analyses.

Primary islets isolation and ex vivo treatment. Wistar rats (Envigo) 27 to 31 days of age

were euthanized, and islets were dissociated from pancreatic tissue as described above. 100 islets/dish were cultured in RPMI 1640 media containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (islet media) for 24 h and then in islet media containing 10% heat-inactivated FBS (hi-FBS) for an additional 24 h. Islets were then cultured for indicated lengths of time with 50 ng/ml IFNγ or vehicle in islet media containing 10% hi-FBS and then harvested in Trizol for mRNA extraction.

Lipid analysis. Monophasic lipid extraction of total cell lysates was performed in methanol/chloroform/water (2:1:0.74) [150]. Non-targeted lipidomics were performed using an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, CA). Full scan MS spectra were acquired at a resolution of 100,000 in both positive and negative ionization modes using direct infusion nano-electrospray ionization (nESI). Higher Energy Collisional Dissocation (HCD) tandem mass spectra were collected on lipid ions of interest using the Orbitrap analyzer at 100,000 resolution. nESI parameters, Orbitrap inlet and S-lens settings, peak finding, lipid identification and normalization against internal standards were as previously described [151]. Data are fold changes in lipid species abundance relative to control.

Lipid droplet staining. INS-1 cells were plated on borosilicate coverslips and treated with IFNγ or vehicle (control) as described above. Cells were fixed in 10% formalin and stained with Oil Red O (0.7%, Sigma) and hematoxylin. Coverslips were mounted with Aqua Mount (Lerner Laboratories). Images were captured with a Nikon DXM1200 microscope at 100X magnification and processed with Nikon ACT-1 software, version 2.20.

Real time quantitative PCR. Total RNA was extracted with Trizol (Invitrogen, CA) and converted to cDNA using High Capacity cDNA synthesis kit (Applied Biosystems, CA). Real-Time PCR was performed using SYBR Green and quantified on a 7500 Real Time PCR system

(Applied Biosystems). Gene expression was calculated as fold change relative to cyclophilin mRNA levels and compared to control samples using the $2^{-\Delta\Delta Ct}$ method. Primers are listed in ESM Method.

Immunoblotting. Total proteins from INS-1 cells were extracted in RIPA lysis buffer (BioRad, CA), and protein levels were determined by Lowry assay. Equal amounts of protein from individual samples were resolved by electrophoresis using gradient (4-20%) or 10% SDS polyacrylamide gels (BioRad). Proteins were transferred onto Immubilon-PSQ PVDF membranes (MilliporeSigma). The membranes were blocked in 7% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were probed with primary antibodies (1:1,000 dilution) overnight at 4^{o}C, then washed with TBS-T and incubated with secondary antibodies (1:10,000 dilution) for 1 h at room temperature. Immunoreactivity was quantified using Odyssey Imaging system (LI-COR Bioscience, NE) and analyzed by Image Studio Lite (version 4.0) software. β-actin or tubulin were used for loading control. Primary antibodies: rabbit polyclonal anti-G0S2 (Proteintech, IL), rabbit polyclonal anti-ATGL (Cayman Chemical), rabbit monoclonal p-STAT1 Y701 (Cell signaling, MA), goat polyclonal anti-actin (Santa Cruz, CA), mouse monoclonal anti-tubulin (Sigma) were used at 1:1000 dilution. Secondary antibodies: Alexa Fluor® 680 goat anti-rabbit IgG, Alexa Fluor® 800 goat anti-mouse IgG (Invitrogen, CA), IRDye 800CW donkey anti-goat (LI-COR Biosciences) were used at 1:10,000 dilution.

PIC transfection. INS-1 cells were incubated for 12 h with IFNγ or vehicle as described above, except that serum-free INS-1 media containing 0.5% BSA was used to facilitate PIC transfection. Cells were then incubated for 12 h with PIC (2.5 μg/ml; Sigma) that was complexed with lipofectamine 2000 (1:2 ratio; Thermo Fisher). C75 (20 μM; Sigma) or cerulenin (40 μM;

Cayman Chemical, MI) was added 1 h before PIC transfection to inhibit fatty acid synthase (FASN). Cells were harvested for mRNA analysis.

*Statistical analysis***.** Data analysis were performed using GraphPad Prism version 7.0 (La Jolla, CA). Data are mean ± SEM of minimum of three animals or *in vitro* experiments. Comparisons between groups were performed by two-tailed unpaired Student's *t* tests or one-way ANOVA with Tukey's post-hoc correction. $p < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Changes of neutral lipid levels in LEW1.R1 rat islets prior to insulitis

To initiate β cell autoimmunity, juvenile LEW.1WR1 rats (32-37 days of age) were injected (i.p.) with 1 μg/g PIC every other day for up to 12 consecutive days (**Fig. 2-1*A***). PIC, a dsRNA viral mimetic and TLR3 agonist, has been shown to induce insulitis and subsequently T1D after 11 to 34 days of treatment in both male and female LEW.1WR1 rats [146]. Treatment of juvenile LEW.1WR1 rats with PIC led to increased appearance of islet-associated (peripheral and infiltrating) monocytes (CD68+ cells) at 6 (grade 1.1, n=7), 8 (grade 1.5, n=7) and 12 days (grade 2.7, n=7), and T cells (CD3+ cells) at 8 (grade 1.5, n=3) and 12 days (grade 1.9, n=5). The temporal increase in islet-associated CD68+ and CD3+ cells preceded the loss of fasting blood glucose control (i.e. diabetes) that began at 12 days (**Fig. 2-1*B***). To investigate the impact of early ß cell autoimmunity on islet lipid levels prior to marked invasion of islet infiltrating immune cells, LEW.1WR1 were treated with PIC for 4 days after which islets were isolated 48 h after the last PIC injection (4D+48h pi) and lipidomic analyses were performed. Strikingly, islets from PIC-treated LEW.1WR1 rats had significantly elevated TAG (~16.5-fold, p=0.04), whereas there were no significant differences observed for total phospholipid (PL), diacylglyceride (DAG), cholesterol esters (CE), or non-esterified fatty acids (NEFA) (**Fig. 2-1*C***). The changes in islet

TAG occurred independently of any notable changes in plasma lipid levels (data not shown).

2.3.2. The association of IFNγ signaling with alteration of lipid composition in LEW.1WR1 islets

Susceptibility of LEW.1WR1 rats to PIC- and viral-induced T1D, in part, lies within the major susceptibility locus *Iddm37* near MHC that contains the IFNγ-regulated diubiquitin (ubiquitin D, *Ubd*) gene [147,148] and is dependent on type 1 interferon signaling [149]. Gene expression studies revealed that 16 h post-injection of PIC (6D+16h pi) there were significant increases in expression of interferon regulatory factor 7 (*Irf7*) and *Ubd*, indicative of a strong type 1 (IFNα, IFNβ) and type 2 interferon (IFNγ) response, respectively (**Fig. 2-1*D***). Forty-eight hours post-injection of PIC (4D+48h and 6D+48h) *Irf7* mRNA levels were markedly reduced, while the reduction in *Ubd* mRNA levels was less pronounced. These data suggest that interferons released during initiation of PIC-induced T1D in LEW.1WR1 rats might regulate islet TAG metabolism and this served as the impetus to explore the impact of IFNγ on lipid metabolism in INS-1 cells.

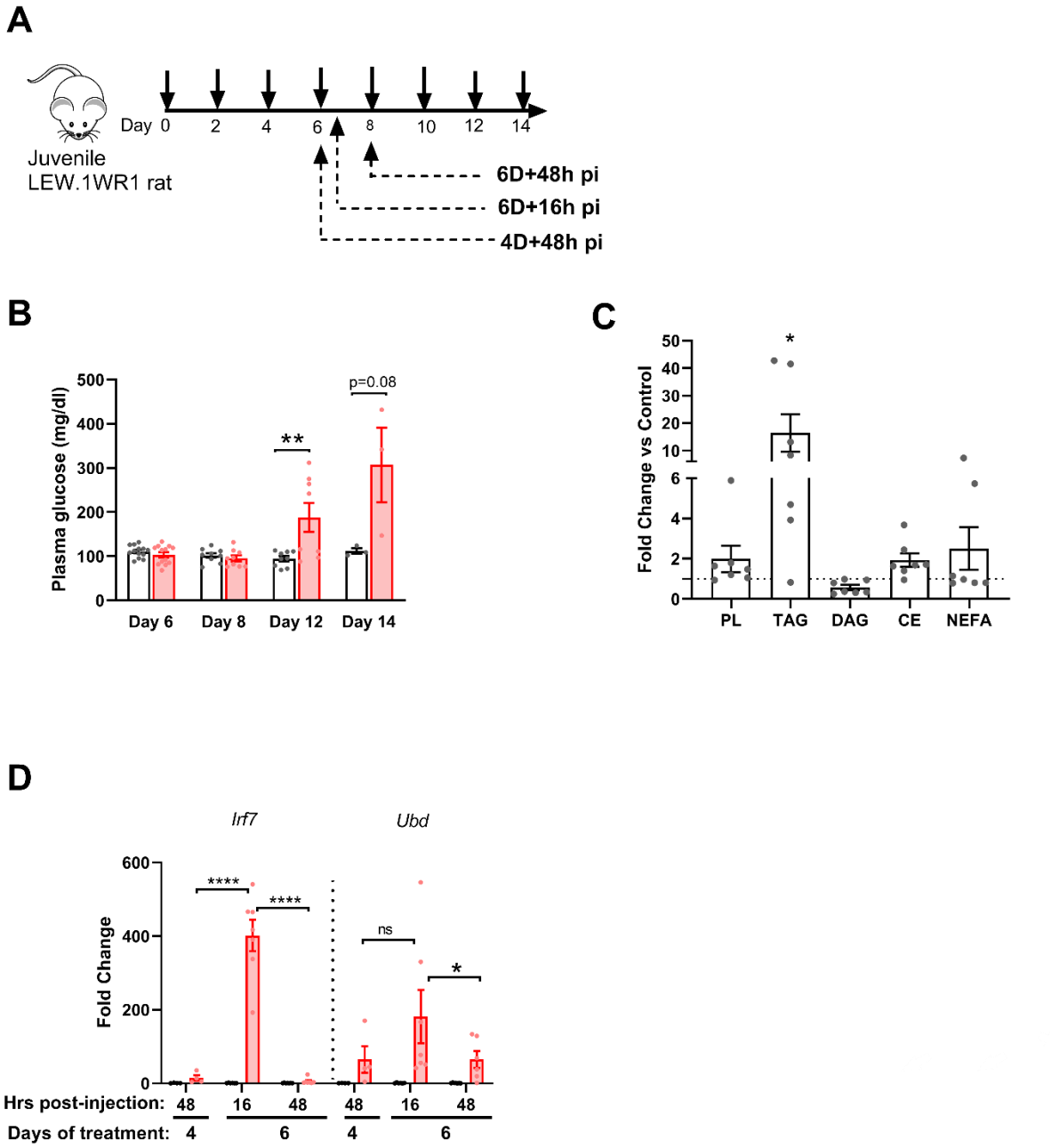


Figure 2-1. Treatment of LEW.1WR1 rats with PIC *in vivo* increases triacylglyceride levels and IFN signaling in pancreatic islets.

(**A**) Timeline of intraperitoneal injections of PIC (solid arrows) and islet isolation (dashed arrows), pi: post injection. (**B**) Plasma glucose levels 6, 8, 12 and 14 days after PIC treatment. Results are mean ± SEM (n = 13 for 6D, n = 8 for 8D and 12D, n = 3 for 14D). Control: white bar; PIC: red bar. $^{**}p < 0.01$, by unpaired Student's t-tests. (**C**) Relative levels of phospholipid (PL), triacylglyceride (TAG), diacylglyceride (DAG), cholesterol ester (CE) and NEFA in islets isolated from LEW.1WR1 rats treated for 4 days and isolated 48 h post last injection (4D+48h pi). Data are fold change in ion abundance relative to control, shown as mean ± SEM (n = 7). Dash line

Figure 2-1 (cont'd)
represents the control level for each lipid species. *$p < 0.05$ vs. control islets, by unpaired Student's t-tests. (**D**) Levels of interferon regulatory factor 7 (*Irf7*) and diubiquitin (*Ubd*) mRNA in islets isolated from LEW.1WR1 rats at 4D+48h pi, 6D+16h pi and 6D+48h pi. Results are mean ± SEM ($n = 7$). Control: white bar; PIC: red bar. *$p < 0.05$, ****$p < 0.0001$, ^{ns}not significant by multiple unpaired t-tests with Tukey's correction.

2.3.3. The effect of IFNγ on neutral lipid levels in pancreatic β cells INS-1

To investigate the ability of IFNγ to regulate β cell lipid metabolism, INS-1 cells were treated with 50 ng/ml IFNγ for 6 to 24 h, after which cellular lipid composition and gene expression were examined via non-targeted lipidomics and qPCR, respectively. The classical effects of IFNγ were confirmed by robust phosphorylation of Signal Transducer and Activator of Transcription 1 (STAT1) within 30 mins (**Fig. 2-2*A***) and sustained upregulation of target genes *Stat1* and *Ubd* throughout 24 h time course (**Fig. 2-2*B***). IFNγ significantly decreased cellular TAG levels at 6 h (32%, $p = 0.03$), whereas treatment for 24 h led to a significant increase in TAG levels (~ 1.5-fold, $p = 0.02$) (**Fig. 2-3*A***). IFNγ treatment for 6 h trended to decrease CE levels and this reached significance (40%, $p = 0.02$) at 24 h (**Fig. 2-3*B***). In INS-1 cells, TAG makes up the most neutral lipids (data not shown) and is stored in LDs. It was hypothesized that IFNγ also regulates LD in a biphasic manner. Indeed, treatment with IFNγ for 6 h reduced both the size and numbers of LDs compared to control cells (**Fig. 2-3*C***). In contrast, treatment with IFNγ for 24 h resulted in increased numbers and size of LDs and they tended to cluster in the perinuclear region. Collectively, the results indicate that IFNγ regulates neutral lipid metabolism, especially TAG, and LDs in a temporal manner in pancreatic β cells.

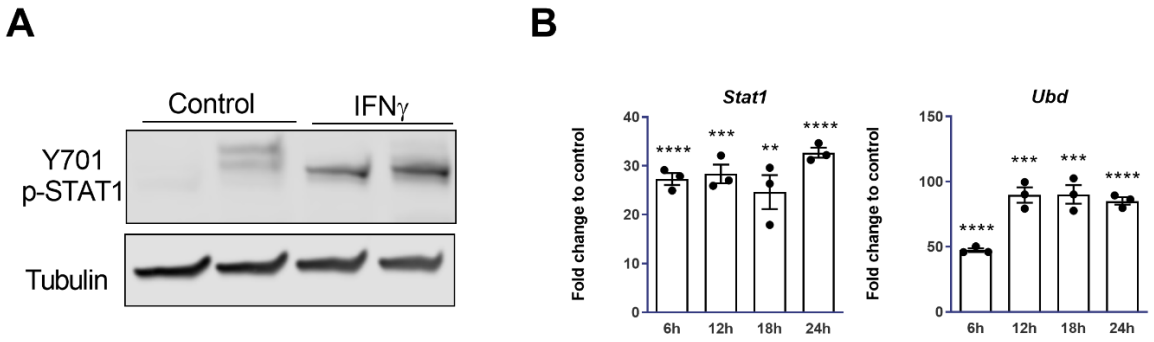


Figure 2-2. IFNγ induces STAT1 phosphorylation and expression of classic STAT1/IFNγ target genes in INS-1 cells.
(A) Phosphorylation of STAT1 in INS-1 cells treated with IFNγ for 30 mins. Blot is a representative image from one experiment performed in duplicate. **(B)** mRNA levels of *Stat1* and *Ubd* in INS-1 cells treated with IFNγ for 6,12, 18 and 24 h relative to control at each time point. Results are mean ± SEM (n = 3); ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. control, by unpaired Student's t-tests.

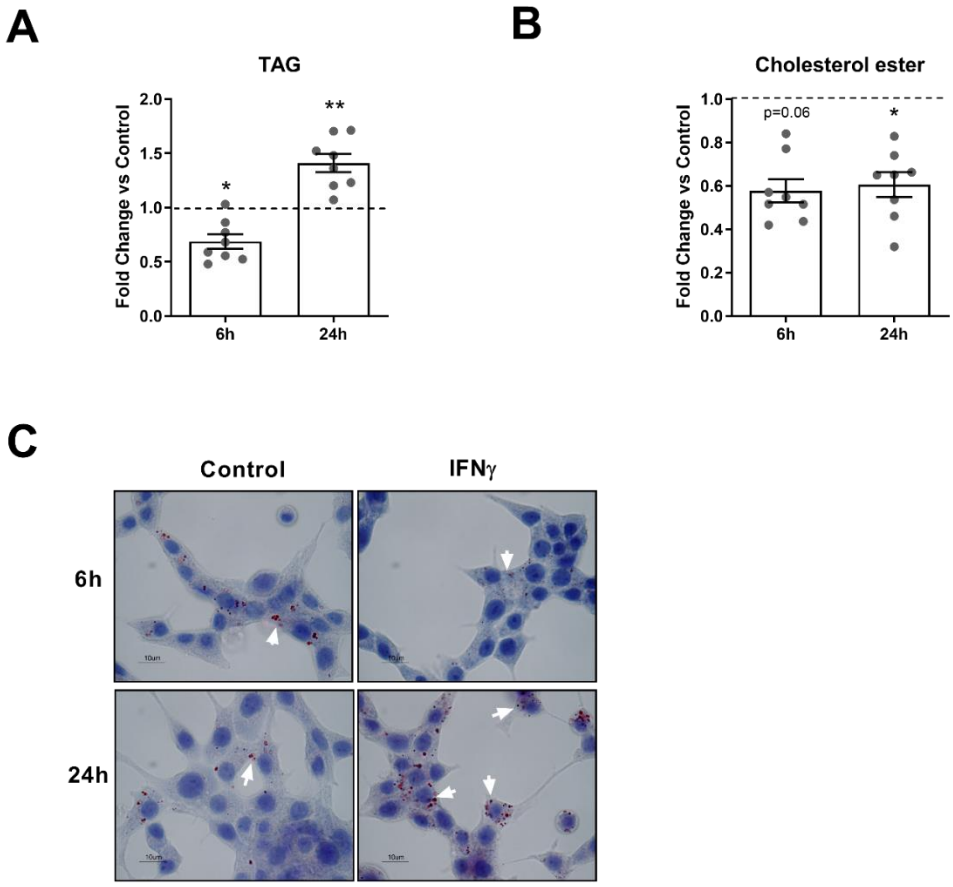


Figure 2-3. IFNγ temporally regulates neutral lipid and lipid droplet levels in INS-1 cells.
(A-B) Relative levels of TAG (**A**) and cholesterol ester (**B**) in INS-1 cells treated with IFNγ for

Figure 2-3 (cont'd)
6 or 24 h. Data are fold change in ion abundance relative to control for each time point, shown as mean ± SEM (n = 8). *p < 0.05, ** p < 0.01 vs. control for each timepoint, by unpaired Student's t-tests. **(C)** Representative Oil-Red O staining of INS-1 cells treated with IFNγ for 6 or 24 h. Arrows are pointed at LDs. Images are representative of three independent experiments. Scale bar = 10 μm.

2.3.4. IFNγ - mediated temporal regulation of TAG lipolysis and LD formation

To investigate the mechanisms whereby IFNγ temporally regulates TAG and LD levels in INS-1 cells, expression levels of key genes involved in TAG lipolysis and LD metabolism were measured during a 24 h time course. IFNγ significantly increased mRNA levels of *Pnpla2*, which encodes the rate-limiting enzyme adipose triglyceride lipase (ATGL) of TAG lipolysis [21], early at 6 h of treatment (2.2-fold, p = 0.0005) and this was sustained out to 24 h (3.3-fold, p = 0.00007) (**Fig. 2-4*A***). In a similar manner, IFNγ induced ATGL expression from 12 h (2-fold, p = 0.002) to 24 h (2-fold, p = 0.009) (**Fig. 2-4*B***). In contrast, IFNγ did not alter mRNA levels of *Lipe* (Fig. 4*A*), which encodes hormone-sensitive lipase (HSL) and catalyzes the last two steps of TAG lipolysis [21]. ATGL activity is controlled by two regulators: G0S2, which binds to ATGL at rest and prevents ATGL from being recruited to LD membranes, and CGI58, which binds to ATGL and activates it under stimulation of lipolysis [21]. IFNγ did not alter mRNA levels of *Abdh5*, i.e. CGI58 (**Fig. 2-4A**). Interestingly, IFNγ significantly suppressed *G0s2* at 6 h (40%, p < 0.0001), whereas *G0s2* mRNA levels were restored to baseline by 18 h and upregulated at 24 h (1.8-fold, p = 0.04) (**Fig. 2-4*A***). G0S2 protein levels followed a similar time course with IFNγ reducing G0S2 levels at 6 h (60%, p = 0.02) while G0S2 levels are restored to baseline by 24 h (**Fig. 2-4*B***). Noticeably, the restoration and upregulation of *G0S2* is likely not due to negative feedback through lipolysis, as blocking ATGL with Atglistatin did not block IFNγ-induced expression of *G0s2* at 24 h (**Supp. Fig. 2-1**).

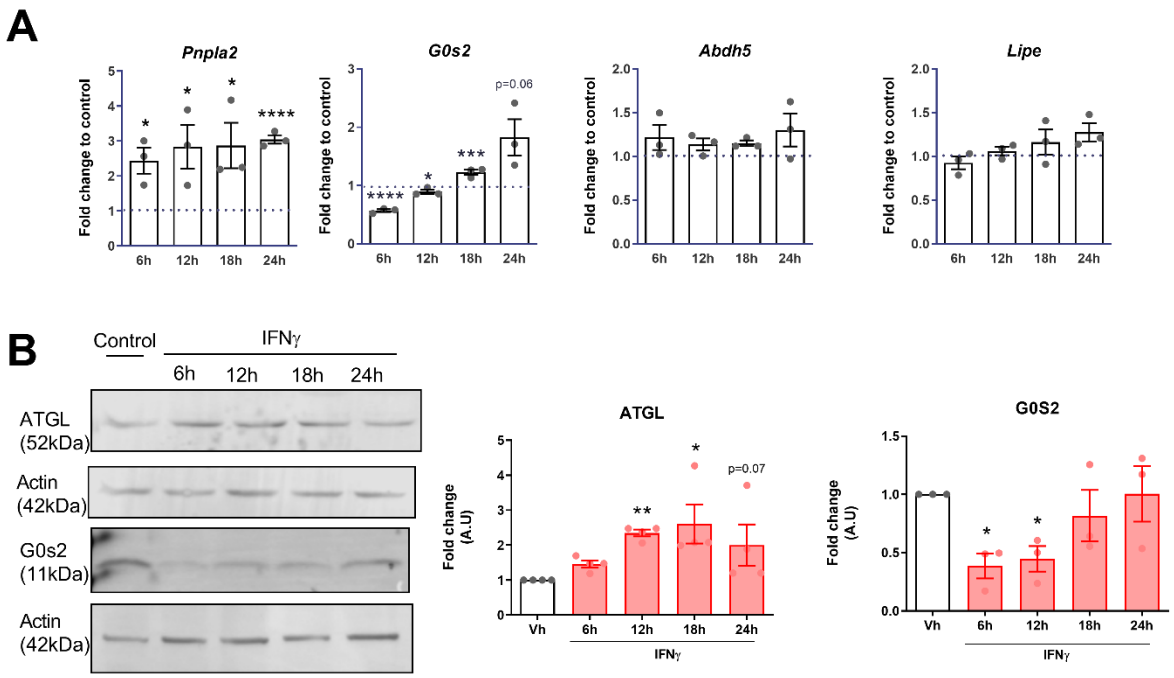


Figure 2-4. IFNγ regulates lipolysis in a biphasic manner in INS-1 cells.
(**A**) mRNA levels of genes regulating TAG lipolysis (*Pnpla2, G0s2, Abdh5* and *Lipe)* in INS-1 cells treated with IFNγ for 6, 12, 18 and 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). *p < 0.05, ***p < 0.001, ****p < 0.00001 vs. control for each timepoint by unpaired Student's t-tests. **(B)** Representative western blot of ATGL and G0S2 protein levels in INS-1 cells treated with IFNγ for 6, 12, 18 and 24 h. Quantitative data are fold change relative to control, shown as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, vs. control by unpaired Student's t-tests.

Lipolysis and incorporation of TAG into LDs are strongly regulated by perilipins family [152]. Treatment with IFNγ significantly increased Plin1 and Plin2 mRNA (~2-fold) from 6 to 24 h (**Fig. 2-5*A***). At early timepoints, IFNγ also increased mRNA levels of fat storage induced transmembrane (*Fitm1*), an important gene for nascent LD budding from the ER [153], yet IFNγ did not affect levels of cell death-inducing DNA fragmentation factor-like effector C (*Cidec*), a gene important for LD fusion and growth (**Fig. 2-5*B***). To test whether IFNγ-mediated increase in TAG depends on the availability of exogenous FAs, INS-1 cells were treated with IFNγ in serum-free media containing BSA or 200 μM palmitate complexed to BSA for 24 h. IFNγ significantly

increased TAG levels in both serum-free media (1.3-fold, p = 0.02) and when supplied with exogenous palmitate (2-fold, p < 0.0001) (**Fig. 2-5*B***). IFNγ modestly downregulated CE levels in serum-free media but significantly increased CE levels in palmitate-containing media (1.5-fold, p = 0.01). Overall, these data suggest that IFNγ enhances the capacity to store exogenous FAs into neutral lipids, particularly TAG at 24 h; however, exogenous FAs is not required for IFNγ-mediated TAG accumulation.

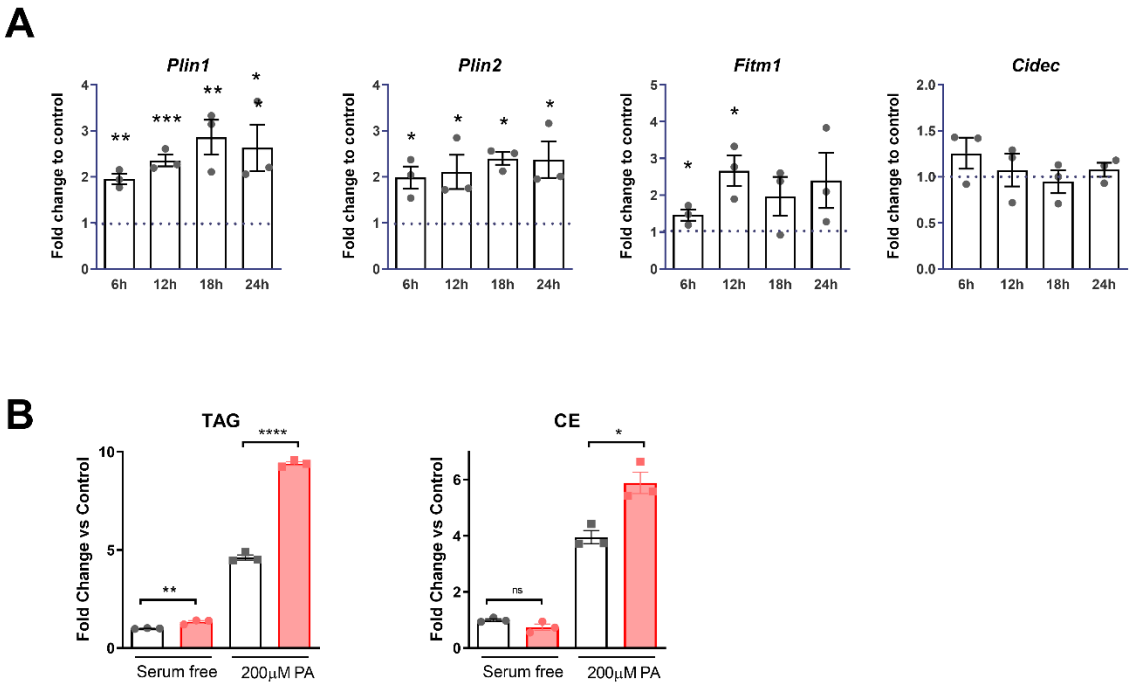


Figure 2-5. IFNγ enhances the expression of LD surface proteins and storage of exogenous fatty acid.
(A) Levels of *Plin1, Plin2, Fitm1* and *Cidec* mRNA in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (*n* = 3); **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. control for each timepoint by unpaired Student's t-tests. **(B)** Relative levels of TAG and CE in INS-1 cells treated with IFNγ for 24 h in serum-free media containing 0.5% BSA or 200 μM palmitate. Data are fold change in ion abundance relative to control in serum-free media, shown as mean ± SEM (*n* = 3). Control: white bar; IFNγ: red bar. ^{ns} not significant, **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 vs. control by unpaired Student's t-tests.

2.3.5. The impact of IFNγ on *de novo* lipogenesis

The increase in TAG observed in IFNγ-treated INS-1 cells cultured for 24h in serum-free

media (**Fig. 2-5*B***) suggests that FA used for TAG synthesis can come from endogenous sources, i.e. *de novo* lipogenesis. Treatment of INS-1 cells with IFNγ for 24 h significantly elevated cellular NEFA levels (2-fold, $p = 0.02$) (**Fig. 2-6*A***). The most abundant forms of elevated NEFA were predominantly stearic acid (18:0), palmitic acid (16:0) and oleic acid (18:1) (the first digit is the number of FA carbons, the second digit is the number of double bonds), which are products of DNL (**Fig. 2-6*B***). Consistent with FA composition, the most abundant forms of TAG increased by IFNγ at 24 h were TAG(52:2), TAG(50:1), TAG(50:2), TAG(52:3), TAG(48:0) and TAG(48:1), which predominately contain 16:0, 16:1, 18:0, 18:1 FAs (**Fig. 2-6*B***). Treatment of INS-1 cells with IFNγ increased the mRNA levels of FA synthesis genes, including acetyl CoA carboxylase α (Accα) at 18 h (1.7 fold, $p = 0.02$) and 24 h (2.1-fold, $p = 0.004$), ATP-citrate lyase (*Acly*) (1.3-fold, $p = 0.001$) and fatty acid synthase (*Fasn*) (1.5-fold, $p = 0.03$) at 24 h (**Fig. 2-6*C***). IFNγ for 24 h also increased the mRNA levels of Sterol response element binding protein 1 c (*Srebp1-c)*, the most important transcription factor for DNL. Interestingly, IFNγ did not affect the expression level of TAG synthesis enzyme diacyl glycerol transferase 1 (*Dgat1*), but downregulated *Dgat2* starting at 12h (~50% reduction at 24h, $p < 0.0001$) (**Fig. 2-6*D***). The increased levels of NEFA is also likely not due to increase FA uptake, as IFNγ decreased expression of lipoprotein lipase (*Lpl*) without altering FA translocase (*Cd36*) (**Supp. Fig. 2-2*A***).

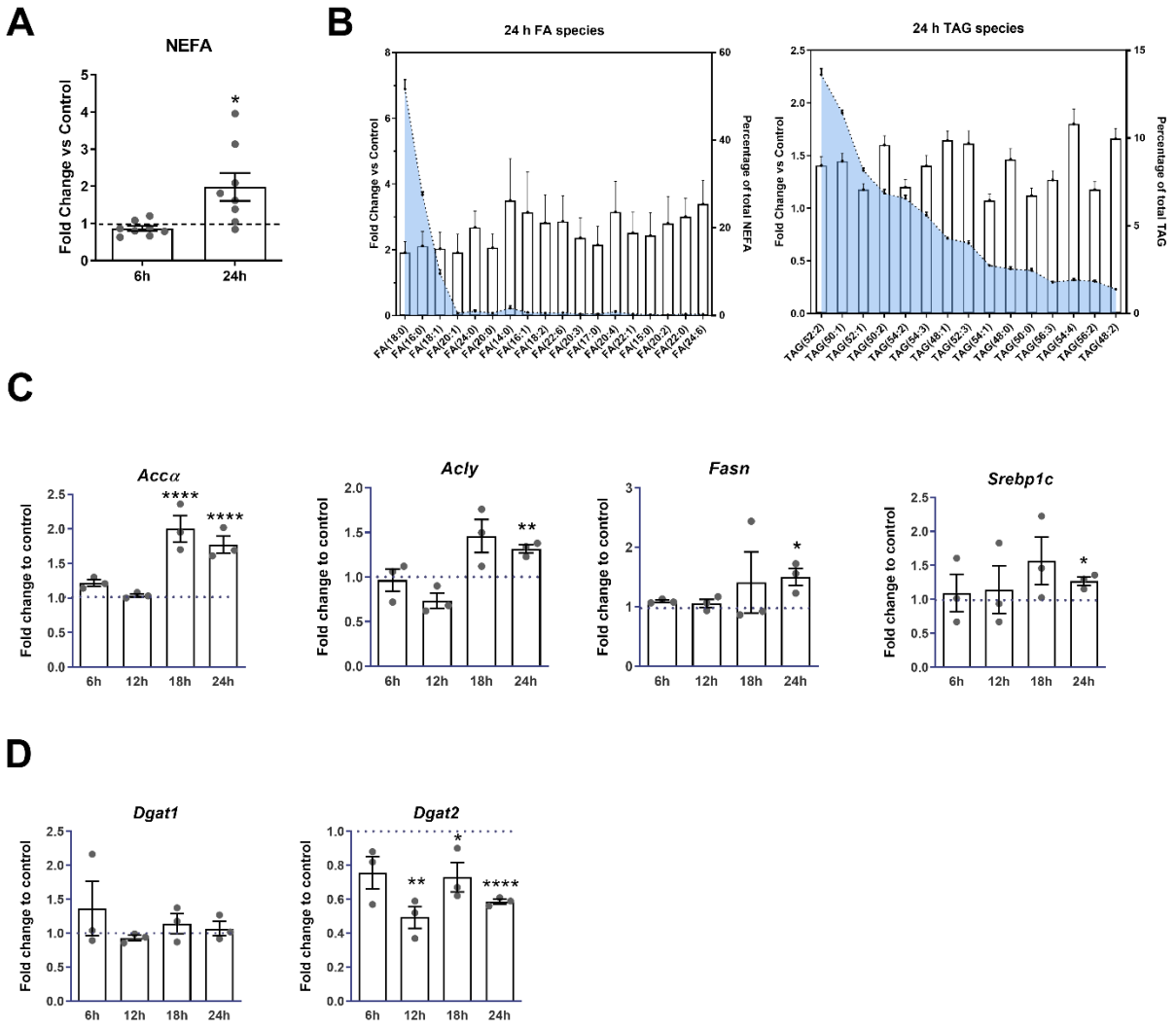


Figure 2-6. IFNγ stimulates *de novo* FA synthesis.
(A) Relative levels of NEFA in INS-1 cells treated with IFNγ for 6 or 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 8). *p < 0.05 vs. control for each timepoint by unpaired Student's t-tests. **(B)** Levels of the most abundant NEFA (left panel) and TAG (right panel) species in INS-1 cells treated with IFNγ for 24 h. Bar graphs indicate fold change relative to control (left Y axis). Shaded areas in blue indicates the relative abundance of each FA or TAG species per total amount of NEFA or TAG in IFNγ-treated cells (right Y axis). Results are mean ± SEM (n = 8). **(C-D)** mRNA levels of genes that regulate *de novo* FA synthesis (*Accα*, *Acly*, *Fasn*, *Srebp1c*) and TAG synthesis (*Dgat1*, *Dgat2*) in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each timepoint, shown as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ****p < 0.0001 vs. control for each timepoint by unpaired Student's t-tests.

Unlike its regulation of NEFA, IFNγ did not alter cholesterol levels at either 6 h or 24 h

(**Fig 2-7*A***), nor the transcription factor of cholesterol synthesis *Srebp2* (**Fig. 2-7*B***). However, IFNγ downregulated mRNA levels of *Soat1/2*, encoding for Sterol-O acyltransferases 1/2, which catalyze CE synthesis from cholesterol and FAs, at early time points (**Fig. 2-7*B***). IFNγ did not regulate the levels of reverse cholesterol transporter ATP-binding cassette *Abca1* (**Supp. Fig. 2-2*B***). These findings support the conclusion that the late effect of IFNγ involves increasing *de novo* FA synthesis, which can be responsible for increase TAG accumulation. In contrast, IFNγ inhibits CE synthesis, causing decreased CE levels.

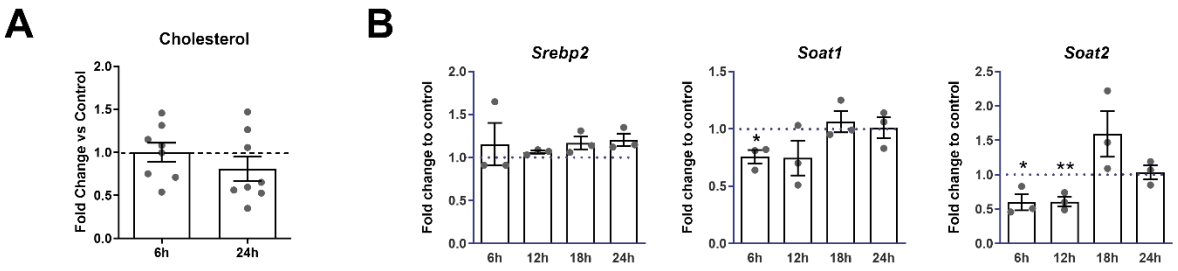


Figure 2-7. IFNγ downregulates cholesterol ester synthesis in INS-1 cells.
(A) Relative levels of cholesterol in INS-1 cells treated with IFNγ for 6 or 24 h. Data are fold change in ion abundance relative to control for each timepoint, shown as mean ± SEM (n = 8). **(B)** Levels of *Srebp2*, *Soat1* and *Soat2* mRNA in INS-1 cells treated with IFNγ for 6 or 24 h. Data are fold change relative to control for each timepoint, shown as mean ± SEM (n = 3). *p < 0.05, ** p < 0.01 versus control for each timepoint, by unpaired Student's t-tests.

2.3.6. Regulation of genes involved in lipid metabolism by IFNγ in primary rat islets

To understand the effects of IFNγ in primary cells, isolated Wistar rat islets were treated with IFNγ for 12 h and 24 h, after that the expression of genes involved in FA and TAG metabolism was examined. Similar to INS-1 cells, IFNγ significantly upregulated *Pnpla2* and downregulated *Dgat2* and *Lpl* early at 12 h (**Fig. 2-8*A***). At 24 h, IFNγ remained to significantly upregulate *Pnpla2*, and trended to downregulate *Dgat2* and *Lpl*, although the difference did not reach significance (**Fig. 2-8*B***). IFNγ did not alter *G0s2*, *Plin2*, *Accα* and *Soat2* in primary islets at both timepoints.

Interestingly, IFNγ significantly downregulated *Fasn* at 12 h. These results indicate that IFNγ regulates similar lipid metabolism pathway in β cell line and islets, however, there are some difference that might be due to the heterogeneity of islets.

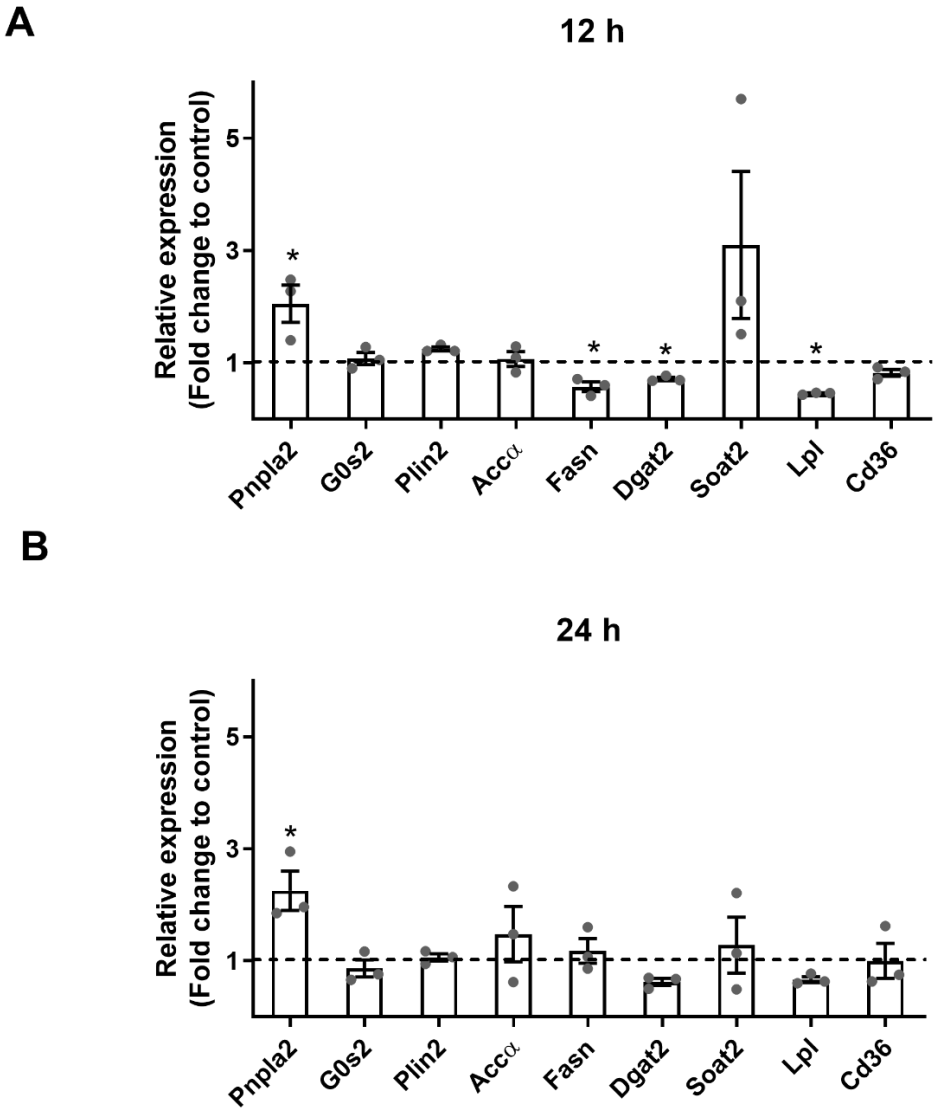


Figure 2-8. IFNγ regulates lipid metabolism genes in primary rat islets.
(**A-B**) Relative levels of lipid metabolism genes in primary rat islets treated with IFNγ for 12 (**A**) or 24 h (**B**). Data are fold change in mRNA expression relative to control, shown as mean ± SEM (n = 6-12). *$p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ versus control, by unpaired Student's t-tests.

2.3.7. The link between IFNγ-induced *de novo* FA synthesis and anti-viral gene expression

Lipids have emerged to play a vital role in host defense mechanism, especially against viral infection [87,88,154]. Thus, IFNγ-mediated increase in DNL, TAG and LDs accumulation was hypothesized to associate with preemptive anti-viral response. To test this hypothesis, INS-1 cells were pretreated with IFNγ for 12 h, then transfected with 2.5 μg/ml PIC for another 12 h, after which mRNA levels of classic anti-viral genes, i.e. interferon beta (*Ifnb*) and Mx dynamin like GTPase 1 (*Mx1*) were examined. IFNγ by itself had modest effect on *Ifnb* and *Mx1* levels compared to PIC which strongly upregulated these genes (**Fig. 2-9*A*** and ***B***). Pretreatment with IFNγ, however, significantly enhanced PIC-induced expression of *Ifnb* (3.9-fold, $p = 0.012$) and *Mx1* (3.5-fold, $p = 0.04$). Strikingly, independent pretreatment with two FASN inhibitors, C75 and cerulenin, completely abolished IFNγ-mediated potentiating effects on *Ifnb* and *Mx1* expression (**Fig. 2-9*A*** and ***B***). Meanwhile, blocking lipolysis with lipase inhibitors orlistat and atglistatin, or stimulating DNL and cholesterol synthesis with LXR agonist TO901317 did not alter IFNγ priming effect (**Supp Fig. 2-3*A*** and ***B***). Additionally, exogenous oleic acid also did not mimic IFNγ-mediated potentiating effects (**Supp Fig. 2-4**). Collectively, these results suggest that *de novo* FA synthesis process induced by IFNγ plays an important role in amplifying the anti-viral gene expression for host defense in β cells.

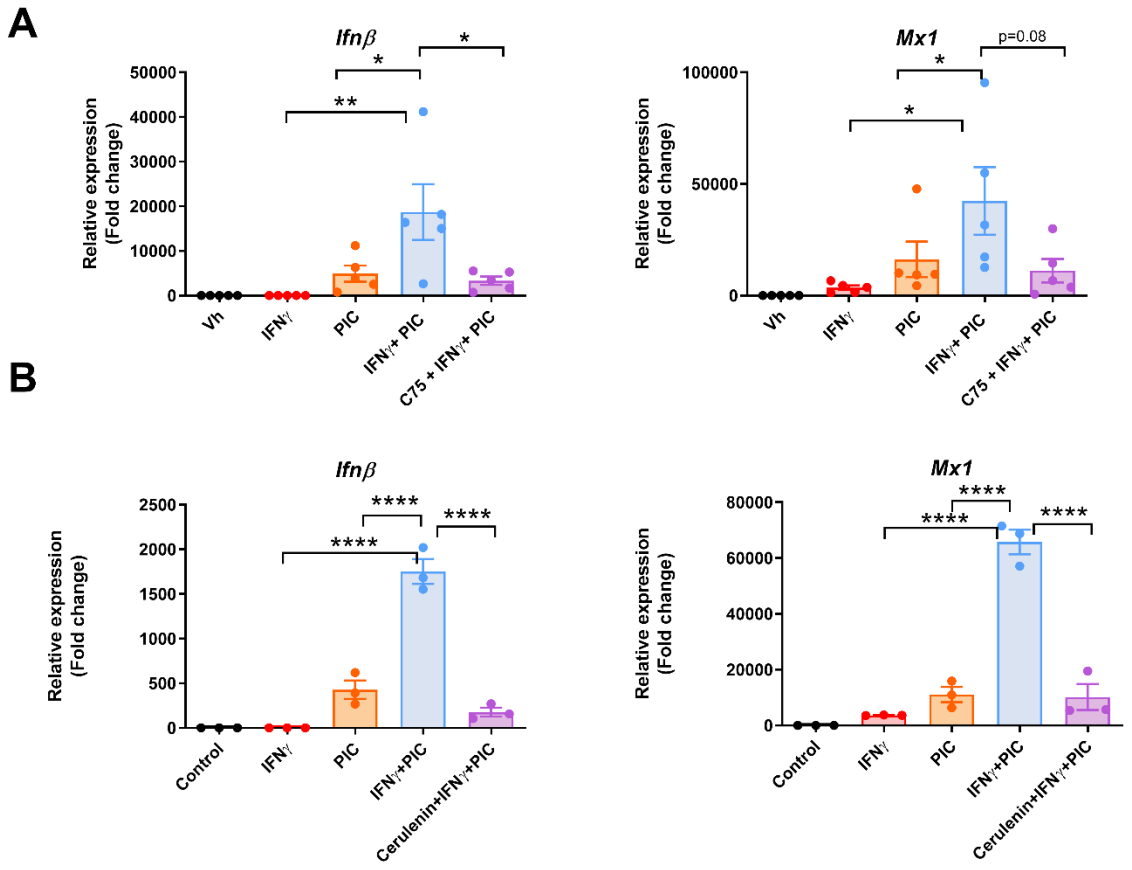


Figure 2-9. IFNγ-induced changes in lipid metabolism are associated with a priming effect for anti-viral gene expression.
(A-B) Expression levels of anti-viral genes (*Ifnß*, *Mx1)* in INS-1 pretreated with IFNγ for 12 h and then transfected with 2.5 µg/ml PIC for 12 h. To inhibit *de novo* FA synthesis, C75 (5 mg/ml) **(A)** or cerulenin (40 mg/ml) **(B)** was added 1 h prior to PIC transfection. Results are mean ± SEM (n = 5 in **(A)** and n = 3 in **(B)**); *p < 0.05, **p < 0.01, ***p < 0.001, ****p <0.0001, by one-way ANOVA with multiple comparison and Tukey's correction.

2.4. Discussion

Studies reported herein illustrate that inflammatory signals, particularly anti-viral interferons, cause pancreatic islets and β cell to undergo substantial changes in cellular neutral lipid metabolism. In a type 1 diabetic rodent model, induction of β cell autoimmunity led to elevated islet TAG level that precedes insulitis and diabetes and corresponds to a prolonged IFNγ signaling. IFNγ was shown to elicit complex effects on pancreatic β cell lipid metabolism: a transient lipolysis, followed by enhanced DNL and accumulation of NEFA, TAG and LD levels

in vitro. Importantly, blocking DNL abrogated IFNγ-priming effect on anti-viral gene expression. Findings from this study suggest that IFNγ alters pancreatic β cell lipid metabolism, especially FA and TAG, that are associated with β cell regulation of host defense.

Acute effect of IFNγ on pancreatic β cell metabolism: transient TAG lipolysis

IFNγ was previously reported to induce lipolysis in isolated adipocytes or systemically *in vivo* at 16 h [41] or 90 mins [155] after treatment. Our results suggest that IFNγ also causes a transient lipolysis in pancreatic β cells, shown as decrease in total TAG and number of LDs at 6 h. The mechanism for this acute effect by IFNγ includes the early upregulation ATGL and downregulation G0S2, the rate-limiting enzyme for TAG lipolysis and its negative regulator, respectively. The effects of IFNγ on *Pnpla2* was also confirmed in primary islets. The burst in lipolysis may serve several important roles. First, lipolysis-derived FAs can be oxidized in mitochondria to generate ATP needed to mount certain response such as activation of gene transcription. Second, lipolysis also plays active roles in innate immunity. Monoglycerides and free FAs were suggested to have direct antimicrobial function [156]. It was suggested that TNFα-induced adipocytes lipolysis creates free saturated FAs that acts as ligands for Toll like receptor 4 (TLR4) and activate neighboring macrophages [157]. Palmitate was shown to activate TLR4 pathway in pancreatic β cells and chemokine production for recruitment of inflammatory monocytes [158]. It is possible that IFNγ-induced lipolysis is responsible to liberate FAs needed to activate innate immune response either within β cells or surrounding immune cells. Alternatively, the bulk supply of long chain FAs derived from lipolysis was shown to be used for phospholipid synthesis necessary for poly virus replication [159]. Whether IFNγ-induced lipolysis is a competitive mechanism to impede viral use of cellular LD storage or to activate receptor signaling requires further investigation.

Late effect of IFNγ on pancreatic β cell metabolism: elevated NEFA, accumulation of TAG and LD clusters

Compared to previous studies showing that IFNγ stimulates lipolysis and attenuates lipid storage in adipocytes [36,45,47], our results are the first to demonstrate that in pancreatic β cells, lipolysis occurs acutely, and TAG accumulation results from long term (24 h) exposure to IFNγ. This can be due to (1) suppressed lipolysis or (2) increased TAG synthesis. First, the upregulation of *G0S2* mRNA and restoration of protein levels to baseline by IFNγ at 24 h suggests a reverse in ATGL inhibition and therefore lipolysis. G0S2 is a crucial regulator of lipolysis in HeLa cells [160], adipocytes [161], hepatocytes [162] and skeletal muscles [163]. Our findings strengthen these studies and imply an important role of G0S2 in IFNγ-mediated effect in pancreatic β cells. Second, the increase in TAG level at 24 h by IFNγ is also possibly due to the late increase in DNL. Notably, IFNγ upregulated *de novo* FA synthesis genes, e.g. *Srebp1*, *Accα*, *Acly*, *Fasn* and increased NEFA levels, particularly 16:0, 16:1, 18:0, 18:1, implying increased flux of FA into storage. Unlike the dynamic regulation of FA and TAG metabolism, IFNγ showed no effect on cholesterol level and cholesterol synthesis regulator *Srebp2*, yet decreased CE levels and *Soat1/2*, suggesting decreased CE synthesis. Notably, IFNγ-mediated effects on CE is reversed only in the presence of exogenous palmitate. These observations suggest that IFNγ preferably enhances storage of FAs into TAG but not CE under regular nutrient condition or with limited FAs. Alternatively, the increase in NEFA might result from decreased CE synthesis and downregulation of *Dgat2*, which is linked to esterification of *de novo* synthesized FA to TAG, whereas *Dgat1* is involved in recycling of TAG and FA re-esterification [164].

In INS-1 cells, the majority of neutral lipids are TAG, which is stored along with CE in phospholipid monolayer-coated LDs. As IFNγ temporally regulates TAG metabolism, IFNγ also

regulates LD numbers in the same manner: a decrease at 6 h, followed by an increase at 24 h. IFNγ sustainably upregulated genes that regulate lipolysis and LD formation, i.e. *Plin1, Plin2, Fitm1* from 6 h to 24 h. In adipocytes, PLIN1, and to the lesser extent PLIN2, are lipolytic barriers that prevent lipase access to the LDs at basal conditions and promote lipolysis under adrenergic activation. FITm1 is an ER protein that is responsible for nascent LD budding from ER membrane. The observation that *Plin1, Plin2* and *Fitm1* were strongly upregulated by IFNγ during the time-course of 24 h suggest a constant enhancement of LD formation, either through breakdown of large LDs during early lipolysis or formation of nascent LDs due to DNL. IFNγ-treated cells showed increased number of LD clusters around the perinuclear region, however, IFNγ did not alter *Cidec* (also known as *Fsp57*), a gene known to enhance LD fusion and clustering [165]. Although the physiological role of these clusters is unknown, they were found in HCV-infected hepatocytes [166], suggesting that IFNγ-mediated effect on LD morphology might link to its associated anti-viral function.

IFNγ-mediated de novo lipogenesis and its link to anti-viral function

IFNγ is an anti-viral proinflammatory cytokine that plays a central role in the pathogenesis of β cell autoimmunity in T1D, yet the link between IFNγ-mediated lipid metabolism in β cells and IFNγ-induced effects are not understood. We demonstrated that in INS-1 cells, IFNγ robustly potentiated PIC-induced expression of anti-viral genes *Ifnb* and *Mx1*, and this priming effect is abolished by inhibiting fatty acid synthesis with C75 or cerulenin. These data suggest that IFNγ-mediated enhancement in DNL, TAG and LD is associated with anti-viral function of IFNγ on pancreatic β cells. This phenomenon could be explained by multiple mechanisms. Carroll et al. proposed that *de novo* FA synthesis provides aceto-acetyl CoA which is necessary for cholesterol synthesis and formation of lipid draft for TLR4 activation in macrophages. They showed that

blocking the formation of this intermediate by C75 abrogated the activation [82]. Although it is unlikely that IFNγ-induced DNL promotes cholesterol synthesis in INS-1 cells, we cannot rule out the possibility of DNL-derived metabolite to amplify TLR3 signaling upon PIC infection. In addition, many anti-viral genes, including type 1 IFNs and IFN-stimulated genes, are regulated through the mitochondrial anti-viral signaling protein (MAVS) or stimulator of interferon genes (STING) pathways upon viral dsRNA recognition. MAVS is localized to a number of membranes, including the mitochondria and mitochondria-associated ER membrane (MAM). Its dimerization is the crucial step for the activation of viral sensor retinoic acid-inducible gene I (RIG-1)-like receptors (Vazquez and Horner, 2015), and was shown to be dependent on lipids [167]. Similar finding also suggests a role of lipid in regulating STING pathway [84]. It is probable that IFNγ-induced DNL serves to enhance the synthesis mitochondria-associated organelles and activation of mitochondria-associated proteins, that leads to its "priming" effect on these signaling pathway upon PIC infection. However, further studies are required to examine the effect of IFNγ-mediated lipid change to MAVS recruitment to mitochondria and its activation. Finally, FAs can have direct anti-viral effects. Many viruses rely on FAs, especially very long chain FAs and saturated FAs to produce virion envelops [168]. Long chain polyunsaturated FAs (PUFA) were shown to have antibacterial and antiviral function, especially arachidonic acid (20:4), DHA (22:6) and EPA (20:5) [169]. PUFAs levels were also increased by IFNγ along other FA species in INS-1 cells, implying a potential mechanism for the anti-viral function of IFNγ.

TAG and LDs are increasingly recognized for their active roles in host-pathogen interaction. Certain HIV anti-viral drugs exert their mechanism via increasing TAG synthesis [170], and inhibiting TAG synthesizing enzyme *Dgat1* also prevented HCV viral replication [171]. LD serves as platform for anti-viral proteins, e.g. viperin [87], and viral multiplication [88,154,172]. Although

the effects on LDs number varies between viruses, LD structure changes markedly during viral infection, signifying its active involvement in host defense mechanism. Reducing LDs content by limiting serum was shown to delay host response to dsRNA by downregulating type 1 IFN production and response in HeLa cells [88]. It was recently shown that IFNγ-induced LD formation in *Mycobacterium tuberculosis* infected macrophages is pivotal for macrophage activation to prevent bacterial acquisition of host lipids [89]. In our studies, IFNγ-mediated priming effect on *Ifnb* and *Mx1* expression could be owing to the enhancement of TAG level and LD clusters formation. However, whether the formation of LDs plays an active role in immune function, or just provides a buffering capacity for NEFA is the question for future research in our lab.

Linking the effect of IFNγ to lipid biomarkers in T1D

Several pre-clinical and clinical studies have shown that metabolic changes in plasma or islets occur before islets autoimmunity. Pre-diabetic children have decreased levels of phosphotidylcholine and TAG in serum before emergence of islet autoantibodies [173]. There is a declined level of sulfatide and expressions of genes regulating sphingolipid metabolism in T1D patient islets. Importantly, polymorphisms of these genes correlate with islet autoimmunity, and artificially increasing sulfatide synthesis in a rodent model completely prevents T1D [174]. Herein, *in vivo* results showed that injection of LEW.1WR1 rats with PIC for 6 days resulted in significant accumulation of TAG in islets 48 h post injection. This alteration occurred before the infiltration of immune cells into the islets, so-called insulitis, and loss of glucose control. This result suggests that pancreatic islets undergo metabolic reprogramming which precedes β cell dysfunction and cell death. In agreement with this interpretation, fibroblasts and peripheral blood mononuclear cells from T1D patients were shown to accumulate more TAG in response to oleic acid or TNFα [175]. Our *in vivo* results suggest an association between IFN signaling and islet TAG levels. We

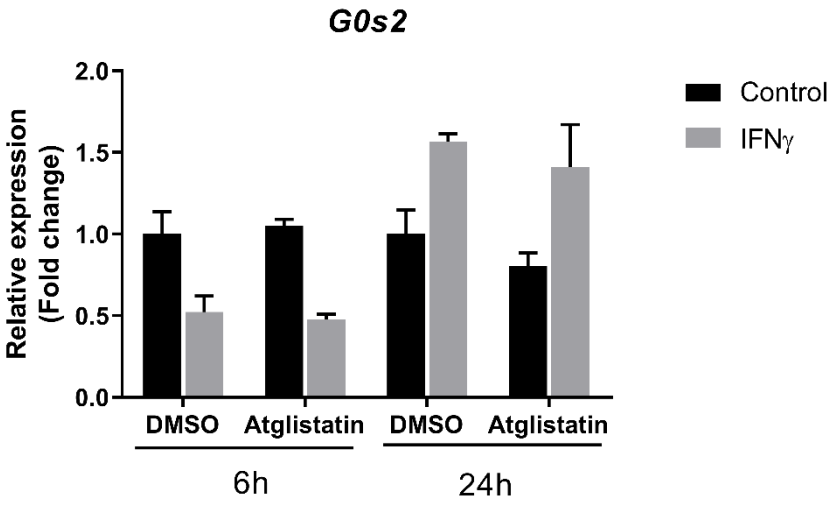
found a strong induction of IFNγ-induced classic gene *Ubd* [176] in islets of PIC-treated rats. *Ubd* is a susceptibility gene for autoimmune diabetes in LEW.1WR1 rats, and deletion of *Ubd* substantially reduced diabetes after viral infection [147], suggesting IFNγ signaling plays a major role in the progression to T1D in this model. Although PIC infection has been known to induced production of both type 1 and type 2 IFN [177], the decrease of *Irf7* compared to sustained level of *Ubd* 48 h after PIC injection prompted us to hypothesize that IFNγ signaling contributes to alteration of lipid composition and autoimmunity of the islets. In combination with the results of *in vitro* studies, it is possible that IFNγ contributes to the alteration of islet lipid metabolism, leading to accumulation of TAG. Further studies with IFNGR $^{-/-}$ LEW.1WR1 rats will be needed to address the direct involvement of IFNγ in regulating pancreatic islets lipid metabolism.

In summary, this chapter shows that in pancreatic β INS-1 cells, IFNγ transiently induces TAG lipolysis, which is followed by an increase in *de novo* FA synthesis and increase in TAG and LD accumulation. The metabolic changes in late phase are associated with an IFNγ-mediated priming effect for anti-viral gene expression. *In vivo* studies in LEW1.WR1 rats also implicate a role of IFNγ in pancreatic β cell metabolism prior to insulitis, shown as a substantial change in TAG level in pancreatic islets following PIC infection. This chapter highlights the importance of cytokine-induced lipid metabolism in pancreatic β cell function in response to environmental insults, e.g. viral infection. These observations have many implications for research into the development of lipid-based immunomodulators to modify cytokine effects for treatment of T1D and other inflammatory diseases.

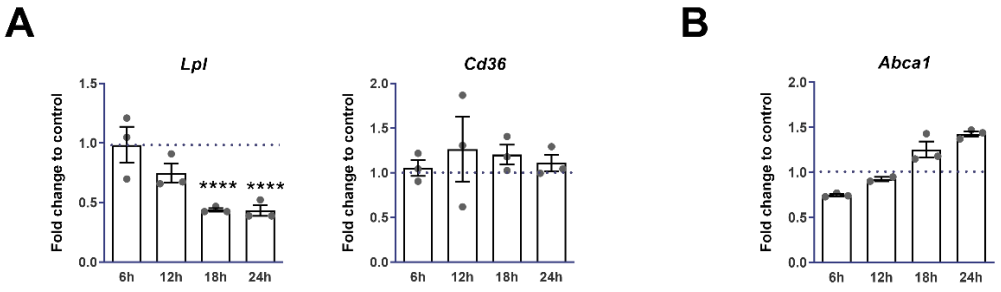
APPENDIX

Supplemental Table 2-1. PCR primer sequences.

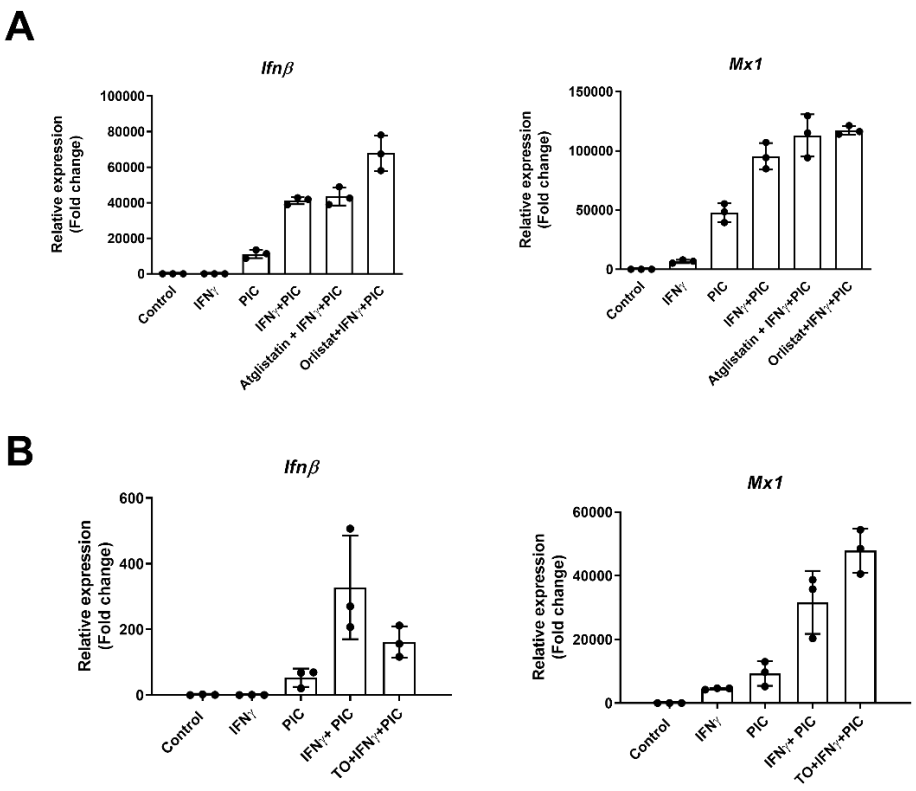
Gene	Protein name	Forward primer (5'-3')	Reverse primer (5'-3')
Abca1	ATP-binding cassette transporter A1	AGCAGTTTGTGGCCCTCTTGT	TGAAGTTCCAGGTTGGGGTACTTG
Abdh5	Comparative gene identification 58	TCTCGCACAACATGTCTAGTAAG	GGCCTATCAGTGCTTAGATCTTC
Acca	Acetyl CoA carboxylase α	TCTCTGATCCACCTCACAGTTGAC	CAGTCAGCAGAGGAACTCTGTACC
Acly	ATP citrate lyase	CATCAAGAAGGCAGACCAGAAG	CATCCCACCAGTATTCCCAATC
Cd36	Fatty acid translocase	AGATTTGTTCTTCCAGCCAACGCC	AGGCTTTCCTTCTTTGCACTTGCC
Fasn	Fatty acid synthase	GTGCACCCCATTGAAGGTTCC	GGTTTGGAATGCTGTCCAGGG
Cidec	Cell death inducing DFFA-like effector protein	CCCAGAAGCCAACTAAGAAG	GGCTTGCATACTGAAGAGAG
Fitm1	Fat storage inducing transmembrane 1	ACACCTTCCTCCTTACCTTCT	CCACCACCTTGTGGGTATATTG
G0s2	G0/G1 switch gene 2	TGATAGCAGAAGGCAAGACAC	AAACAGCATGTGACTCTCTCTC
Ifnb	Interferon beta	ACTACAAGCAGCTCCAGTTC	TGAGGTTGAGCCTTCCATTC
Lipe	Hormone sensitive lipase	TACACAAATCCCGCTATGTGGCCT	AAAGAAGAGCACTCCTGGTCGGTT
Lpl	Lipoprotein lipase	TCTTAGGGTACAGTCTTGGAG	CCGCATCATCAGGAGAAAG
Mx1	MX Dynamin Like GTPase 1	CAGAAGTACGGAGCAGACATAC	CCGGCTTTCTTCCCATGATA
Plin1	Perilipin 1	GCTGTCTCCTCCACCAAAG	CCACAGTGTCTACCACGTTATC
Plin2	Perilipin 2	TCTGAACCAGCCAACATCTG	AACTGCTCCTTTGGTCTTATCC
Pnpla2	Adipose triglyceride lipase	CGGCATTTCAGACAACTTGCCACT	GCAGGTTGAATTGGATGCTGGTGT
Ppia	Cyclophilin	CTTCTTGCTGGTCTTGCCATTCCT	TGGATGGCAAGCATGTGGTCTTTG
Soat1	Sterol O-acyltransferase 1	GAACTCGTGGCACATTCTCT	TCTGGGCTGTTTGCTCTATG
Soat2	Sterol O-acyltransferase 2	TCTGGGCTGTTTGCTCTATG	GAAGGCGAAGAAGATGAGGAG
Srebp1	Sterol regulatory element binding protein 1	GCAGGAAACTGAGAGACCCC	GTACCCACTGGCCTTCTCAC
Srebp2	Sterol regulatory element binding protein 2	CACTCACGCTCCTCGGTCAC	GGATAAGCAGGTCTGTAGGTTGG
Stat1	Signal transducer and activator of transcription	TGAGTTCCGACACCTGCAACTGAA	AGGTGGTCTCAAGGTCAATCACCA
Ubd	Ubiquitin D	CTTTTCTCACTCGGCCTCTG	GAGACCTTGGTTTGGGACCT
Irf7	Interferon regulator factor 7	GGCAAGTGCAAGGTGTACTG	GCCCAAAACCCAGGTAGA



Supplemental Figure 2-1. The effects of lipolysis inhibitor on IFNγ-mediated regulation of *G0s2* expression.
INS-1 was pretreated with 40 μM ATGL inhibitor Atglistatin for 2 h, then treated with IFNγ for 6 h and 24 h. mRNA levels of *G0s2* were measured. Results are mean ± SD, shown as fold change of control samples treated with DMSO at each time point (n = 3 wells).

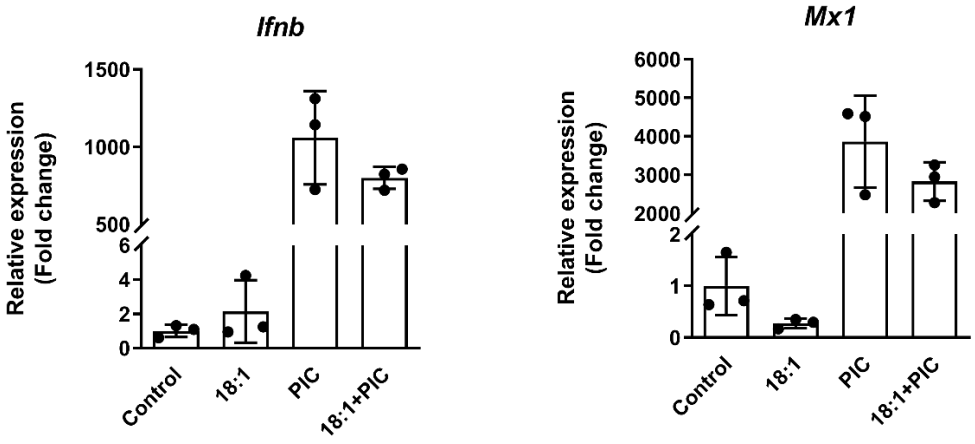


Supplemental Figure 2-2. The effects of IFNγ on expression of genes regulating FA influx and cholesterol efflux.
(**A-B**) mRNA levels of genes involved in exogenous FA uptake (*Lpl*, *Cd36*) and cholesterol efflux (*Abca1*) in INS-1 treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). ****p <0.0001, by unpaired t-tests.



Supplemental Figure 2-3. The effects of lipase inhibitors and LXR agonist on IFNγ-induced anti-viral gene expression.

(**A-B**) mRNA levels of anti-viral genes (*Ifnß, Mx1)* in INS-1 pretreated with IFNγ for 12 h and then transfected with 2.5 μg/ml PIC for 12 h. To inhibit TAG lipolysis, ATGL inhibitor atglistatin (40 μM) or lipases inhibitor orlistat (10 μM) (**A**) was added 1 h prior to PIC transfection. To stimulate *de novo* lipogenesis and cholesterol synthesis, LXR agonist TO901317 (10 μM) (**B**) was added 1 h prior to PIC transfection. Results are mean ± SD (*n* = 3 wells), shown as fold change of gene expression to control cells.



Supplemental Figure 2-4. The effects of exogenous FA on anti-viral gene expression.
mRNA levels of anti-viral genes (*Ifnß, Mx1)* in INS-1 pretreated with 200 µM oleic acid (18:1) for 12 h and then transfected with 2.5 µg/ml PIC for 12 h. Results are mean ± SD (*n* = 3 wells)

Chapter 3. The impact of interferon gamma on beta cell mitochondrial function and endoplasmic reticulum stress

Abstract

Mitochondria and endoplasmic reticulum (ER) govern pancreatic β cell metabolism, cellular homeostasis and play a central role in coupling metabolism and insulin secretion. Overload of free fatty acids, so-called lipotoxicity, disrupts β cell mitochondrial and ER homeostasis, causing ER stress, impaired insulin secretion and ultimately cell death. In chapter 2, IFNγ was shown to transiently induce lipolysis, and later increase DNL and TAG accumulation in INS-1 cells. These changes were associated with the priming effect of IFNγ on anti-viral gene expression. In this chapter, we examined the effects of IFNγ-mediated lipid metabolism on mitochondrial and ER function and their link to insulin secretion. INS-1 cells were treated with IFNγ for 6 to 24 h, then lipid composition, cellular respiration and gene expression involved in mitochondria and ER function were analyzed. Consistent with transient lipolysis and late increase in TAG and LDs, treatment with IFNγ led to an early and sustained elevation of genes regulating long chain FA oxidation (FAO) *Cpt1a, Lcad.* IFNγ, however, caused a time dependent downregulation of *Pgc1a* and upregulation of *Ucp2* and *Plin5,* suggesting a decrease of FAO as FA and TAG accumulate in LDs. Indeed, 24 h exposure to IFNγ treatment resulted in elevated acyl carnitines levels, decreased cellular basal and maximal respiration in response to palmitic acid and glucose, indicating a defect in mitochondrial oxidation capacity. Despite this defect, IFNγ treatment for 24 h had minimal effects on mitophagy and mitochondrial biogenesis, as well as insulin secretion. Next, the impact of IFNγ on ER stress was assessed via measuring gene expression of proteins involved in unfolded protein response. IFNγ did not alter *Chop, Atf4,* and only downregulated *sXbp1* at 24 h. Notably, pretreatment with IFNγ for 24 h enhanced the susceptibility of INS-1 cells to ER stress induced by

other proinflammatory cytokines even in the absence of IFNγ. In summary, these results highlight an intimate link between IFNγ-mediated lipid metabolism and its effects on β cell mitochondrial and ER function. The accumulation of NEFA and TAG due to long term exposure to IFNγ likely increase the susceptibility to cellular dysfunction, via reducing mitochondrial oxidation capacity and potentiating ER stress induced by other proinflammatory cytokines.

3.1. Introduction

Mitochondria and ER are two strongly interconnected cellular compartments that play a central role in regulation of β cell metabolism and insulin secretion. Mitochondria couple glycolysis and FAO with ATP production via oxidative phosphorylation (OXPHOS). In β cells, mitochondrial ATP production is essential for insulin secretion machinery via ATP-dependent closure of potassium channel [178]. ER, on the other hand, is important for synthesis and processing of lipids and proteins, including insulin. Many stimuli, such as excessive nutrients and inflammation, can overwhelm the β cell ER folding capacity, and trigger the unfolded protein response (UPR) to resolve the accumulation of misfolded proteins, resulting in ER stress [179]. As mitochondria-associated ER membrane (MAM) exists between ER and mitochondria, more evidence has proven a strong interconnection between mitochondrial dysfunction and ER stress [180]. The UPR protein CHOP was shown to control mitochondrial metabolic activity [181]. Inducing ER stress with tunicamycin led to increased mitochondrial OXPHOS. This was due to increased Ca2+ transport from ER to the mitochondria, as mitochondria approach perinuclear ER in the early phase of ER stress [182]. Conversely, it was shown that mitochondrial dysfunction activates AMPK, resulting in increase NO production and leads to ER stress in β cells [183].

Long term exposure to saturated FAs and proinflammatory cytokines are widely known for their detrimental effects on mitochondrial and ER function, leading to β cell demise in type 1 and

type 2 diabetes. Saturated FAs activate ER stress in β cells through several mechanisms including protein palmitoylation [184] and depletion of Ca^{2+} [185]. Lipotoxicity also causes mitochondrial oxidative stress [137], limits membrane permeabilization [186] and enhances mitochondrial fission and fusion in islets and β cells [187,188]. Inflammation and proinflammatory cytokines, on the other hand, activate ER stress in NO dependent- and independent- mechanisms [189]. The effects of proinflammatory cytokines on β cell mitochondria are less understood: they were shown to induce ROS production [190], and impair pyruvate oxidation [191]. Understanding the impact of proinflammatory cytokines on β cell mitochondrial function, especially oxidation capacity and bioenergetics is necessary to explain for their mechanism of actions in causing β cell dysfunction.

IFNγ is a pivotal player when combined with other proinflammatory cytokines, e.g. TNFα and IL-1β, to impair β cell ER and mitochondrial homeostasis. IFNγ possesses "priming effects" on macrophages by enhancing the responsiveness to TLR agonists and TNF. In chapter 2, it was demonstrated that IFNγ causes transient lipolysis, followed by an increase in *de novo* lipogenesis in INS-1 cells. The increase in DNL by IFNγ was shown to be necessary for amplifying the gene expression for anti-viral response. It is hypothesized that IFNγ-mediated fluctuation in lipid levels modifies β cell mitochondrial and ER function. Particularly, the late accumulation of NEFA and TAG resulted from IFNγ treatment can potentially predispose β cells to cellular stress. In this chapter, we investigated the temporal actions of IFNγ on mitochondrial and ER function of INS-1 cells, focusing on FAO and UPR, and whether it contributes to insulin secretory function and β cell susceptibility to ER stress induced by other cytokines.

3.2. Materials and methods

Cell culture. INS-1 cells (passage 71-84) were cultured in RPMI 1640 media (Gibco, MD) containing 11.1 mM glucose and supplemented with 10% fetal bovine serum (FBS) (Atlanta

Biologicals, GA), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 50 µM 2-mercaptoethanol (Sigma), 1 mM sodium pyruvate (Sigma), and 10 mM HEPES (Sigma) (complete media) at 37^{o}C in a humidified incubator containing 95% air and 5% CO_2. In all experiments, cells were seeded at a density of $0.25x10^6$ cells/cm^2. Twenty-four hours after plating, cells were washed and incubated for an additional 24 h in complete media containing 10% heat-inactivated FBS (hi-FBS). Cells were then treated with IFNγ or vehicle (0.0005% BSA/PBS) for indicated amount of times in complete media containing 10% hi-FBS. At the end of the experiments, cells were washed once with PBS and harvested in Trizol and lysis buffer for mRNA and protein analysis as described below. For lipidomic analysis, cells were washed once with PBS then quickly frozen in liquid nitrogen and stored at -80^{o}C before processing for lipid extraction.

cDNA synthesis and real time PCR. Total RNA was extracted with Trizol (Invitrogen). RNA was converted into cDNA using High Capacity cDNA synthesis kit (Applied Biosystems, CA). cDNA template was amplified for qPCR with SYBR green mastermix (Applied Biosystems) follow the manufacturer's instruction and detected by Applied Biosystems® 7500 Real Time PCR system. Gene expression is calculated as fold change relative to cyclophilin mRNA levels and compared to control samples using the $2^{-\Delta\Delta Ct}$ method.

Lipid extraction and Tandem Mass spectrometry. Monophasic lipid extraction from total cell lysate was performed in methanol/chloroform/water (2:1:0.74) [150]. Non-targeted lipidomics assays were performed using an LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific). Full scan MS spectra were acquired at a resolution of 100,000 in both positive and negative ionization modes using direct infusion nano-electrospray ionization (nESI). Higher Energy Collisional Dissocation (HCD) tandem mass spectra were collected on lipid ions of interest using the Orbitrap analyzer at 100,000 resolution. nESI parameters, Orbitrap inlet and S-lens

settings, peak finding, lipid identification and normalization against internal standards were as previously described [151]. Data are reported as normalized abundances of lipid species relatively to control group to correct for difference in total lipid levels among experiments.

High resolution respirometry. In the assays using palmitate as substrate, $2x10^6$ INS-1 cells were seeded, sub-cultured and treated with IFNγ for 6 or 24 h in INS-1 media as described in Cell culture section. Cells were washed and incubated in glucose-free, serum-free Krebs-Ringer-HEPES buffer (KRHB) (120 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 100 mM HEPES, 25 mM $NAHCO_3$, 0.1% BSA) for 30 min to stimulate the sensitivity to assay substrate [192]. Cells were then detached with trypsin 0.25% EDTA, washed and resuspended in 30 µl of KRHB. Cells were injected into chambers of the Oxygraph (Oroboros, Austria) that contained 2 ml of KRHB containing 200 µM palmitate bound to 0.5% BSA and 0.5 µM carnitine at 37ºC with magnetic stirring. Palmitic acid (MilliporeSigma) was complexed with Probumin® FA-free BSA (MilliporeSigma) as previously described [19]. In the assays using media with different concentration of glucose, INS-1 cells cells treated with IFNγ for 24 h was washed, detached and resuspened in 30 ul of KRBH. Cells suspension were injected into chambers that contained KRBH media with 11.1 mM glucose or 2.8 mM glucose.

In both experiments, cells were first allowed to reach stable oxygen consumption rate (OCR) and recorded basal OCR. Oligomycin (MilliporeSigma), an ATP synthase inhibitor was added to the final concentration of 0.125 µM to block respiration coupled to ATP synthesis. Next, carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (Fisher Acros Organics, NH) was added in increments to uncouple OXPHOS and electron transport, thus induce maximal respiration. After oxygen consumption reached maximum, complex I inhibitor rotenone (MilliporeSigma) was added to the chambers to the final concentration of 2 µM to block mitochondrial respiration. The

corresponding oxygen consumption rates after each addition were calculated as the time derivative trace using DatLab software (Oroboros). Non-mitochondrial was determined as the OCR after the addition of rotenone (OCR_{Rot}). Other components of mitochondrial respiration were calculated as described below [193]

Basal respiration: OCR_{basal}- OCR_{Rot}

Leak respiration: OCR_{oligo}- OCR_{Rot}

ATP-linked respiration: Basal respiration – leak respiration

Maximal respiration: OCR_{CCCP}- OCR_{Rot}

Reserve respiratory capacity: Maximal respiration – Basal respiration

Immunoblotting. Total proteins from INS-1 cells were extracted in RIPA lysis buffer containing protease inhibitors (Sigma), then protein levels were determined by Lowry assay. Equal amounts of protein from individual samples were resolved by electrophoresis using gradient (4-20%) or 10% SDS polyacrylamide gels (BioRad). Gels were transferred onto Immubilon-PSQ PVDF membranes (Millipore). The membranes were blocked in 7% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were probed with primary antibodies (1:1,000 dilution) overnight at 4^{o}C, then washed three times with TBS-T and incubated with secondary antibodies (1:10,000 dilution) for an hour at room temperature. Primary antibodies: rabbit polyclonal COXIV (Cell signaling, MA), goat polyclonal anti-actin (Santa Cruz, CA), rabbit monoclonal anti-LC3B (Cell signaling), rabbit polyclonal anti-p62 (Sigma). Secondary antibodies: Alexa Fluor® 680 goat anti-rabbit IgG (Invitrogen, CA), IRDye 800CW donkey anti-goat (LI-COR Biosciences, NE). Membranes were washed three times with TBS-T and developed by fluorescent detection using Odyssey Imaging system (LI-COR Bioscience). Immunoreactivity was quantified by Image Studio Lite (version 4.0) software. β-actin

was used for loading control.

Citrate synthase assay. INS-1 cells were treated with IFNγ for 6 h and 24 h, after which cells were lysed in Tris-HCl buffer, pH 7.0, and immediately frozen in liquid nitrogen. Citrate synthase activity of whole cell extract was measured according to Oroboros protocol. Briefly, samples were added to a reaction mixture that contains acetyl CoA, 5,5`-dithiobis [2-nitrobenzoic] (DTNB) (Sigma, MO) and oxaloacetate. Citrate synthase catalyzes the reaction between oxaloacetate and acetyl CoA to form citrate and CoA-SH. The reaction capacity of the citrate synthase can be indirectly measured by the reaction of CoA-SH with Ellman reagent (5,5`-dithiobis [2-nitrobenzoic], DTNB) to form a yellow product (5-thio-2-nitrobenzoic, TNB). Kinetic absorbance measurements of samples were performed on a BioTek plate reader at 412 nm for 10 mins at 37ºC. Citrate synthase activity (U/min/mg protein) was calculated as below:

$$\text{Citrate synthase activity} = \frac{\Delta A/\text{min}}{\text{L.}\,\varepsilon.\,(\text{protein amount})}$$

L: pathlength for absorbance measurement.

ΔA/min: change of absorbance per minute, calculated as the slope of kinetic reading

ε: extinction coefficient of TNB at 412. ε =13.6

Cytokine treatment. INS-1 cells were sub-cultured as described above in the Cell culture section, then treated with IFNγ or vehicle in complete RPMI 1640 media containing 10% hi-FBS for 24 h. Cells then were washed, and incubated with 120 pg/ml IL-1β or 25 pg/ml TNFα in complete RPMI 1640 containing 10% hi-FBS. After 12 h, cells were washed and harvested in Trizol for mRNA analysis.

Glucose-stimulated insulin secretion assay. INS-1 cells were seeded in a 12-well plate and sub-cultured as described above in Cell culture section, then incubated with IFNγ or vehicle in complete RPMI 1640 media containing 10% hi-FBS for 24 h. Cells then were washed 3 times with

Krebs-Ringer-HEPES buffer supplemented with 2.8 mM glucose. Cells were then incubated with assay buffer containing 2.8 mM glucose or 16.7 mM glucose for 1 h at 37^{o}C, 95% O_2 for basal and glucose-stimulated insulin secretion, respectively. Insulin release in the media was measured with rat insulin radioimmunoassay (RIA) kit (Millipore).

Statistical analysis. Data analysis were performed using GraphPad Prism (version 7.0). Data are shown as the mean± SEM from at least three *in vitro* experiments with two to three technical replicates unless stated otherwise. Comparisons between groups were performed by unpaired *t* test and corrected for multiple comparisons with Tukey's post-hoc method. Paired t-tests were applied when comparing oxygen consumption rate as the treated and non-treated samples were handled in parallel in each experiment. $p < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. IFNγ-mediated temporal regulation of genes involved in mitochondrial fatty acid oxidation

In Chapter 2, we demonstrated that IFNγ caused a biphasic effect on TAG levels at 6 h and 24 h, suggesting transient lipolysis, followed by suppression of lipolysis. Long term treatment of IFNγ (24 h) also resulted in an increase in NEFA and lipogenic gene expression, indicating increased *de novo* FA synthesis. Thus, it is hypothesized that the FAs released from early lipolysis and synthesized from DNL enhance mitochondrial FAO. Treatment of INS-1 with IFNγ increased acyl carnitine levels at 24 h (3-fold, $p = 0.003$), but not at 6 h (**Fig. 3-1*A***). Acyl carnitines are formed from cytosolic acyl CoA by the action of carnitine palmitoyl transferases 1 (CPT1) on the outer membrane of mitochondria [194]. This is considered the rate limiting step of FAO, as it allows FA to be brought into the mitochondria for oxidation. Acyl carnitines are then converted back into

acyl CoA by CPT2 on the inner membrane [195], and undergo a cascade of β oxidation reactions to generate Acetyl CoA for TCA cycle, and protons for OXPHOS. IFNγ significantly increased mRNA levels of *Cpt1a* and long chain acyl dehydrogenase (*Lcad*) as early as 6 h and maintained until 24 h (**Fig. 3-1*B***). IFNγ modestly, but significantly increased mRNA levels of *Cpt2* from 18 h to 24 h ($p = 0.004$). Unexpectedly, IFNγ significantly downregulated peroxisome proliferator-activated receptor gamma coactivator (*Pgc1a),* a transcriptional coactivator that governs OXPHOS and mitochondrial biogenesis [196], at 18 h ($p = 0.0003$) and 24 h ($p = 0.008$) (**Fig. 3-1*C***). Uncoupling protein 2 (*Ucp2*) is the only UCP expressed in pancreatic β cells, and has been suggested to mediate FA metabolism and negatively regulate insulin secretion [197]. In contrast to *Pgc1a*, IFNγ upregulated *Ucp2* levels in a time-dependent manner starting at 12 h, and reached significance at 18 h ($p = 0.003$) and 24 h ($p = 0.0004$). Finally, IFNγ upregulated *Plin5,* a member of perilipin family important for the lipid exchange between LDs and mitochondria [26], from 12 h to 24 h ($p = 0.02$) (**Fig. 3-1*C***). These data suggest IFNγ sustainably upregulates gene expression involved in long chain FA oxidation in response to increased lipolysis and DNL, and the rise in acyl carnitines can result from increased flux of newly synthesized FAs through the carnitine shuttle into mitochondria. However, long term exposure to IFNγ potentially leads to a defect in mitochondrial FAO and OXPHOS, thus enhancing TAG accumulation in LDs.

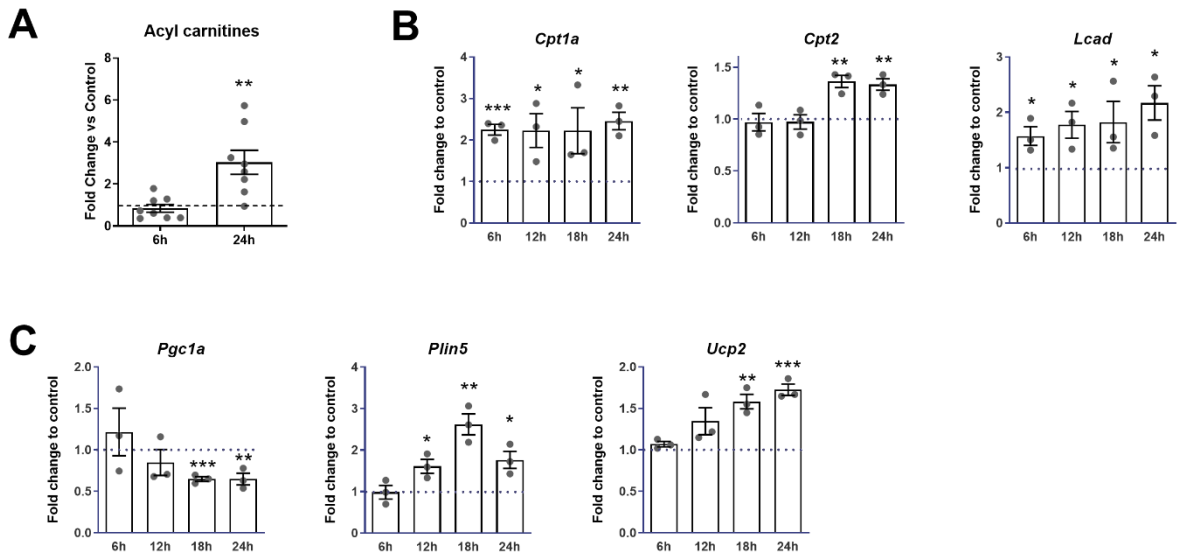


Figure 3-1. IFNγ regulates mitochondrial fatty acid oxidation gene expression.
(A) Relative levels of acyl carnitines in INS-1 cells treated with IFNγ for 6 or 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 8). **(B-C)** mRNA levels of genes regulating mitochondrial FAO (*Cpt1a, Cpt2, Lcad, Pgc1a, Plin5, Ucp2)* in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs. control for each timepoint by unpaired Student's t-tests.

3.3.2. The impact of IFNγ on mitochondrial OXPHOS

The increase in NEFA, LD and acyl carnitine levels at 24 h suggest overload of FAs that exceed mitochondrial oxidation capacity. To investigate the temporal effect of IFNγ on mitochondrial FAO, cellular oxygen consumption rate (OCR) was measured in INS-1 cells treated with IFNγ for 6 h and 24 h. Cells were maintained in glucose-free media for 30 mins to increase their sensitivity to substrate, then cellular respiration was measured in assay containing 200 µM palmitate, 0.5 µM carnitine and no glucose to specifically assess FAO. Treatment of INS-1 cells with IFNγ for 6 h did not affect any components of mitochondrial respiration (**Fig. 3-2*A***, ***B*** and ***E***). In contrast, INS-1 treated with IFNγ for 24 h had significantly reduced basal (p = 0.003), leak (p = 0.002), maximal respiration (p = 0.003) and respiratory capacity (p = 0.04) compared to

untreated cells, suggesting impaired oxidation of FAs (**Fig. 3-2*C, D*** and ***E***). To examine whether long term exposure to IFNγ causes defect in mitochondrial glucose oxidation, we compared the OCR between IFNγ-treated cells and control cells in assay media containing different levels of glucose. Besides reducing ATP-linked respiration when assayed in 11.1 mM glucose (p =0.03) (**Fig. 3-3*A*** and ***B***), IFNγ had no effect on cellular respiration in both conditions (**Fig. 3-3*A*** to ***D***). Collectively, these data suggest that IFNγ decreases the capacity of β cell mitochondria to oxidize FA for OXPHOS, and but has minimal impact on glucose oxidation.

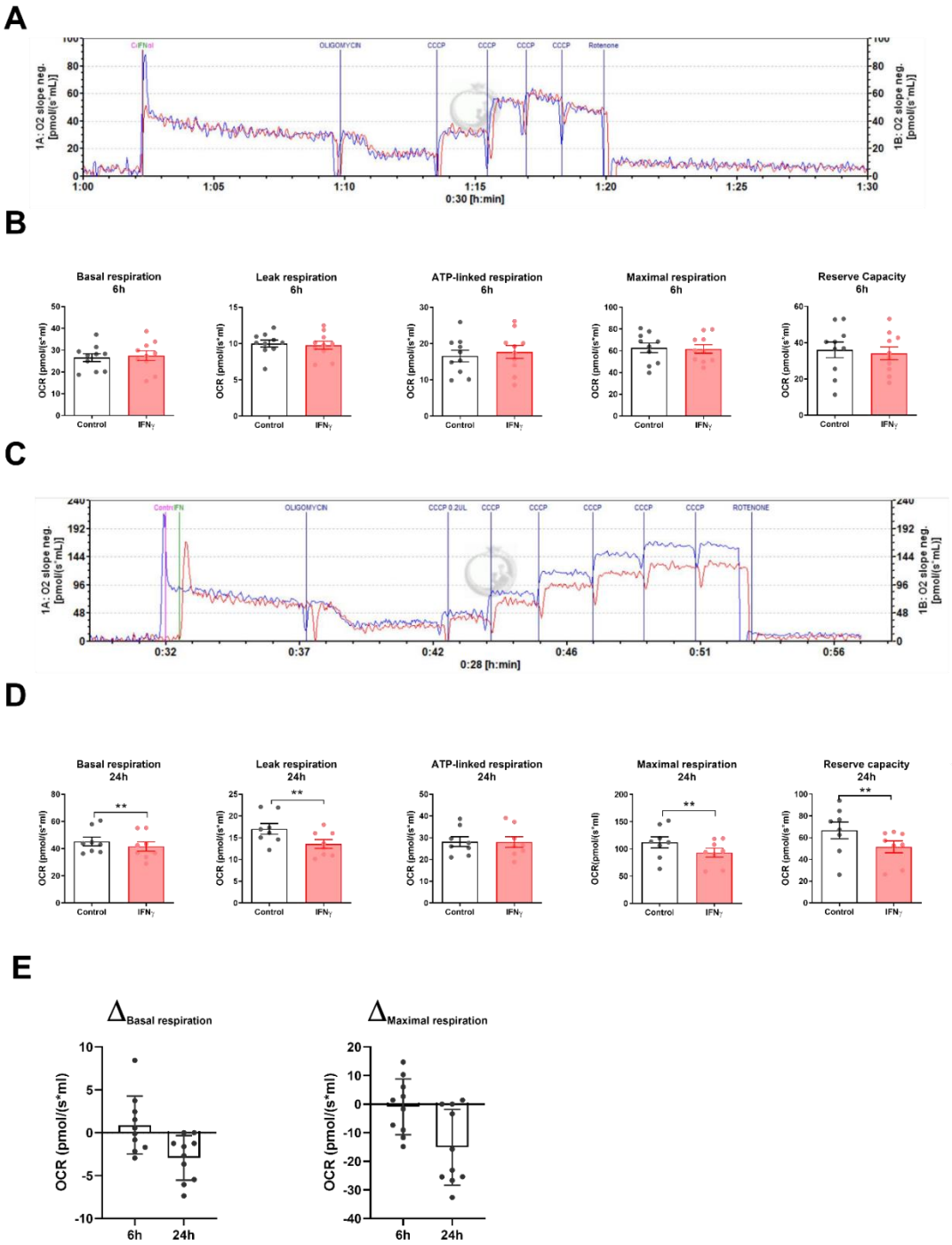


Figure 3-2. IFNγ impairs mitochondrial FAO.

(A, C) Representative respirometry graphs of INS-1 cells treated with IFNγ (red) and control (blue) for 6 h **(A)** or 24 h **(C)** as measured in assay media containing 200 µM palmitate plus 0.5 µM carnitine. Oligomycin was added after the cells reach stable oxygen consumption rate (OCR) to determine ATP-linked respiration and leak respiration. CCCP was titrated to achieve maximum uncoupling respiration. Rotenone was used to inhibit complex I and determined non-mitochondrial

Figure 3-2 (cont'd)
respiration. (**B, D**) Quantified cellular respiration of INS-1 cells treated with IFNγ for 6 **(B)** or 24 h **(D)**. Data are normalized to non-mitochondrial respiration. Results are mean ± SEM (6 h: n = 10, 24 h: n = 8). Control: white bar; IFNγ: red bar. *$p < 0.05$, *$p < 0.01$ by paired Student's t-test. **(E)** Relative difference (Δ) between basal respiration (*left panel*) and maximal respiration (*right panel*) in IFNγ-treated cells vs. control cells at 6 h and 24 h.

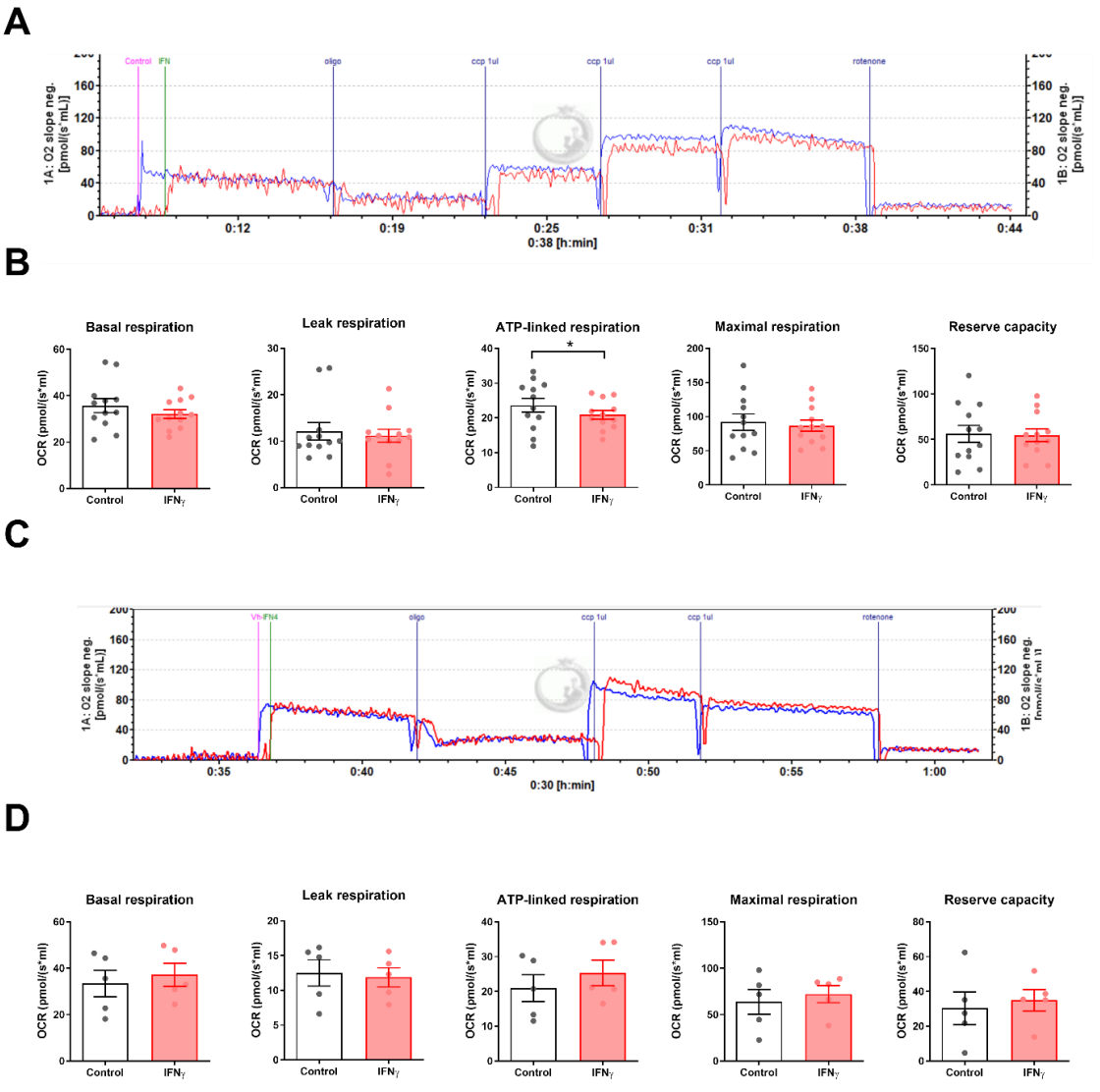


Figure 3-3. IFNγ minimally affects mitochondrial glucose oxidation.
(A, C) Representative respirometry traces of INS-1 cells treated with IFNγ (red) and control (blue) for 24 h as measured in assay media containing 11.1 mM (**A**) or 2.8 mM glucose (**B**). (**B, D**) Quantified cellular respiration of INS-1 cells treated with IFNγ for 24 h measured in assay media containing 11.1 mM (**B**) and 2.8 mM glucose **(D)**. Data are normalized to non-mitochondrial respiration. Results are mean ± SEM (11.1 mM: n = 10, 2.8 mM: n =5). Control: white bar; IFNγ: red bar. *$p < 0.05$, **$p < 0.01$ by paired Student's t-test.

3.3.3. The effect of IFNγ on mitochondrial biogenesis

We have shown that IFNγ caused a decrease in *Pgc1a* mRNA levels and defects in mitochondrial OXPHOS at 24 h. Therefore, it was next tested whether this defect is due to enhanced degradation of mitochondria by autophagy, so-called mitophagy, or reduced mitochondrial biogenesis. IFNγ tended to cause a small time-dependent upregulation of mitofusin 2 (*Mfn2)*, an important gene regulating mitochondrial fusion, however it did not reach significance (**Fig. 3-4*A***). Protein levels of autophagy markers, i.e. autophagosome LC3B-II and autophagic degradation p62 were also examined throughout the 24-hour time course. IFNγ slightly induced the production of LC3B-II and decreased its precursor LC3B-I at 24 h, but did not alter p62 at any time point (**Fig. 3-4*B***). Collectively, the results suggest IFNγ has marginal effects on mitophagy. IFNγ did not alter the mitochondria-specific lipid cardiolipin, or citrate synthase activity at 6 h or 24 h (**Fig. 3-5*A*** and ***B***). Finally, 24 h treatment of IFNγ did not alter levels of cytochrome oxidase (COX) unit 4 (**Fig. 3-5*C***). These data indicate that long term exposure to IFNγ causes a decrease in β cell mitochondrial OXPHOS without changing mitochondrial biogenesis.

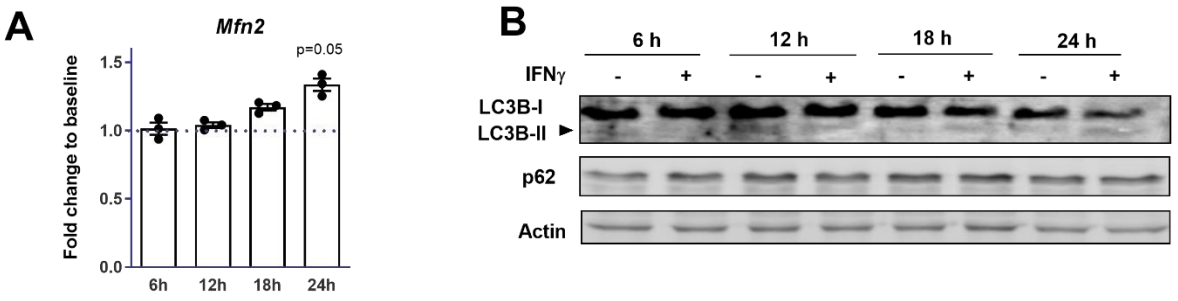


Figure 3-4. IFNγ has minimal effect on mitochondrial fusion and autophagy.
(A) Time-dependent regulation of mitochondrial fusion and mitophagy gene *Mfn2* in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). **(B)** Representative immunoblotting of autophagy markers (LC3B, p62) in INS-1 cells treated with IFNγ for 6 to 24 h (n = 2)

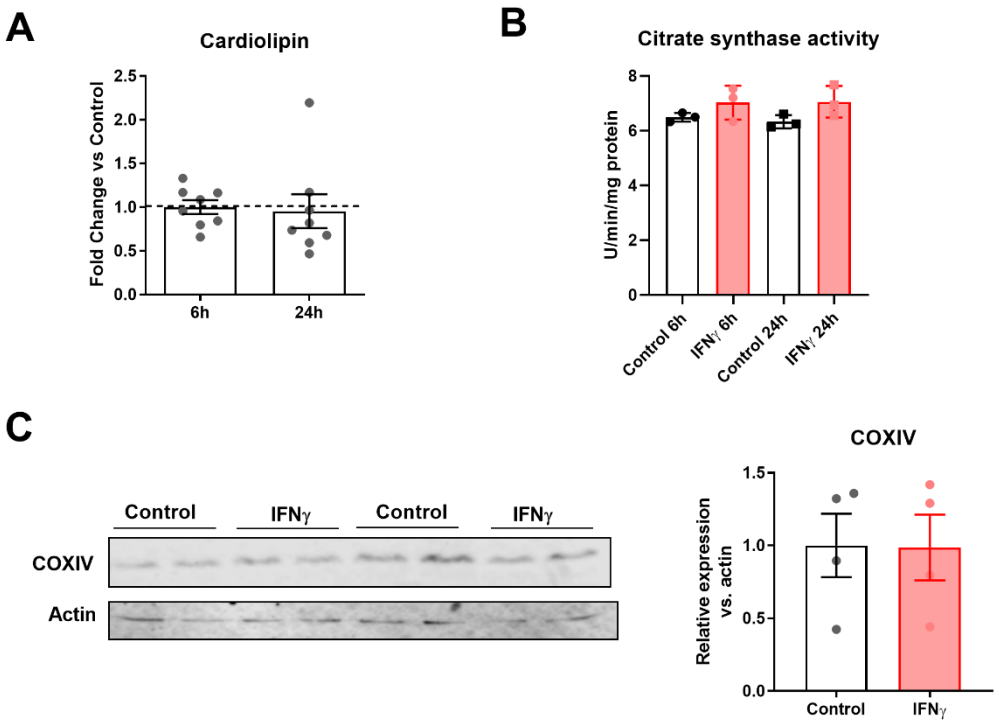


Figure 3-5. IFNγ does not alter mitochondrial biogenesis.
(**A**) Relative levels of cardiolipin in INS-1 cells treated with IFNγ for 6 or 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (*n* = 8). (**B**) Citrate synthase activity of INS-1 treated with IFNγ for 6 h or 24 h, shown as mean ± SD. Bar graphs are representative of two independent experiments. (**C**) Immunoblotting of mitochondrial complex IV subunit (COXIV) in INS-1 cells treated with IFNγ for 24 h. Data are fold change to control, shown as mean ± SEM (*n* = 4). Control: white bar; IFNγ: red bar.

3.3.4. The effect of IFNγ on pancreatic β cell insulin secretion

Since IFNγ caused a decrease in basal and maximal respiration of INS-1 in response to FA and glucose, it was hypothesized that IFNγ would impair glucose-stimulated insulin secretion. In INS-1 cells, IFNγ did not alter *Ins* mRNA levels, suggesting no effect in insulin synthesis (**Fig. 3-6*A***). Next, insulin release from INS-1 cells treated with IFNγ for 24 h was determined in assay media containing 2.8 mM glucose and 11.1 mM glucose for basal and glucose-stimulated insulin secretion (GSIS). Treatment of INS-1 cells with IFNγ for 24 h tended to cause a modest reduction insulin release in response to 2.8 and 16.7 mM glucose, but this was not significant (**Fig. 3-6*B***).

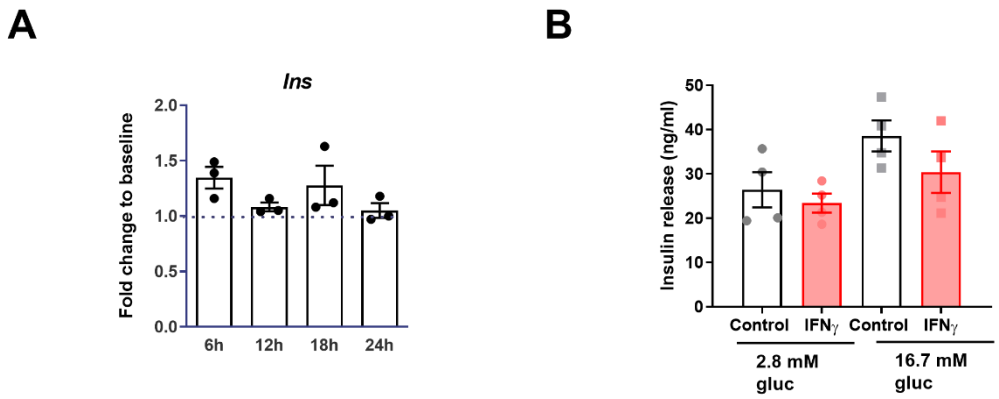


Figure 3-6. IFNγ shows marginal impact on basal and glucose-stimulated insulin secretion. (**A**) Levels of *Ins* mRNA in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). (**B**) Insulin release from INS-1 cells treated with IFNγ for 24 h. Basal and glucose-stimulated insulin secretion were measured at 2.8 mM glucose and 16.7 mM glucose concentration, respectively. Data are mean ± SEM (n = 4).

3.3.5. The effect of IFNγ on unfolded protein response and ER stress

Excessive FAs are known to trigger ER stress in β cells, causing lipotoxicity and cell death. We next investigated whether IFNγ-mediated accumulation of NEFA and TAG at 24 h causes ER stress. IFNγ by itself was insufficient to activate UPR, shown as unchanged levels of *Atf4* and *Chop* at all time points (**Fig. 3-7*A***), yet downregulated *sXbp1* levels by 30% at 24 h (p = 0.0001). Notably, pretreatment with IFNγ for 24 h followed by IL-1β led to a significant increase in *Chop, sXbp1* levels compared to IL-1β alone (p < 0.0001 and p = 0.008, respectively), despite IFNγ was completely washed out (**Fig. 3-7*B***). This priming response by IFNγ also applied with TNFα treatment (**Fig. 3-7*C***). This indicates that IFNγ-mediated lipid accumulation is not adequate to cause ER stress per se but increases the susceptibility of β cells to other cytokine-induced ER stress.

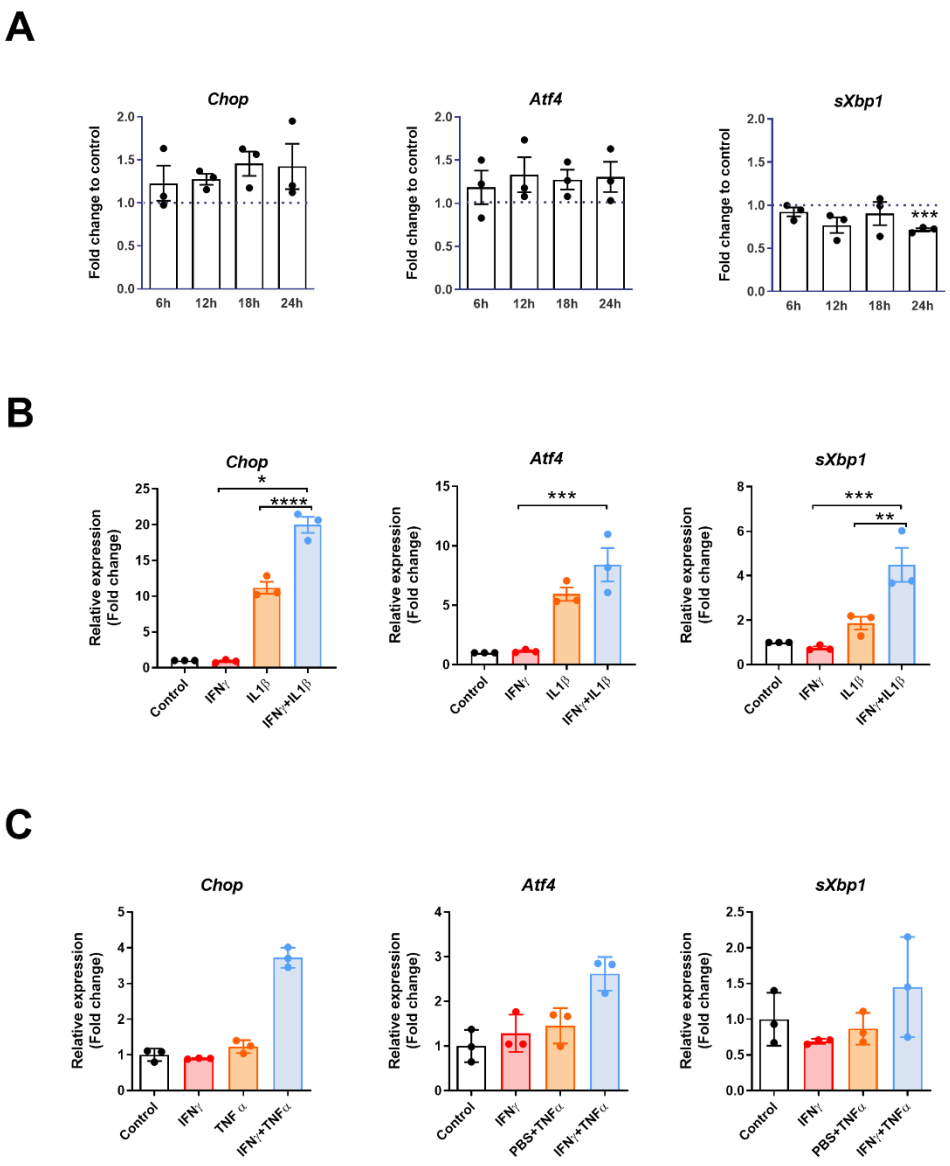


Figure 3-7. IFNγ increases the susceptibility to ER stress induced by other proinflammatory cytokines.

(A) Relative levels of ER stress markers (*Chop*, *Atf4*, *sXbp1*) in INS-1 cells treated with IFNγ for 6 to 24 h. Data are fold change relative to control for each time point, shown as mean ± SEM (n = 3). ****$p < 0.0001$ by unpaired t-tests. (**B**) INS-1 cells were treated with IFNγ for 24 h, washed and treated with 120 pg/ml IL-1β for 12 h. ER stress markers were measured. Data are fold change to control, shown as mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$ between indicated groups, by one-way ANOVA with Tukey's correction for multiple comparison. **(C)** Similar to (B), except INS-1 cells were treated with 25 pg/ml TNFα for 12 h post IFNγ-treatment. Data are fold change to control, shown as mean ± SD of three wells. Results are representative of two independent experiment.

3.4. Discussion

In chapter 2, IFNγ was demonstrated to cause a dynamic change in intracellular FA and TAG levels in pancreatic β cells. In this chapter, we showed that IFNγ-mediated lipid metabolism is associated with sustained upregulation of FAO genes, yet ultimately decreased mitochondrial oxidation capacity. However, IFNγ did not elicit a significant impact on mitochondrial biogenesis and insulin secretion. IFNγ also had minimum effect on ER stress, but increased the susceptibility of β cells to other proinflammatory cytokine-induced ER stress.

IFNγ upregulates mitochondrial FAO gene expression and increases acyl carnitines levels

IFNγ was previously shown to transiently induce lipolysis, then stimulate DNL and cause a biphasic regulation of TAG levels in INS-1 cells. Lipolysis creates free FAs that are transported into mitochondria for oxidation and generation of ATP. IFNγ induced expression of long chain FAO genes, i.e. *Cpt1a* and *Lcad* early at 6 h and it was sustained out to 24 h. Interestingly, the upregulation of *Cpt2* only occurred at 18 h and beyond. Since these FAO genes are regulated by PPAR [198], the increased expression of *Cpt1a*, *Cpt2* and *Lcad* is likely compensatory to oxidize newly formed FAs, particularly long chain FAs from IFNγ-induced lipolysis at 6 h, and increased DNL at later timepoint. IFNγ-induced acyl carnitine accumulation at 24 h could be simply attributed to the increased flux of FAs into mitochondria for oxidation, since we did not see any effect of IFNγ on carnitine transporter *Cact* (data not shown). Elevated plasma acyl carnitines are biomarkers for mitochondrial FAO disorders [199] and metabolic diseases such as diabetes [200,201] and non-alcoholic steatosis hepatitis [202]. IFNγ-mediated acyl carnitine accumulation in pancreatic β cell can be a mechanism for the detrimental effects of proinflammatory cytokines on β cell function in type 1 diabetes.

IFNγ impairs mitochondrial FAO and OXPHOS to enhance lipid storage

In addition to regulating genes involved in mitochondrial carnitine shuttle and β oxidation, IFNγ also upregulates *Plin5* from 12 h to 24 h. The role of PLIN5 in lipid storage and mitochondrial FAO is currently debatable. PLIN5 is largely expressed in oxidative tissues, and was shown to channel FA from LDs to mitochondria for oxidation, in response to increasing energy demand or to protect cell from lipotoxicity in cardiomyocytes and skeletal muscles [26,203]. In our studies, the time-dependent upregulation of PLIN5 and the accumulation of TAG by IFNγ suggest the opposite. Indeed, studies in β cells and islets suggest that PLIN5 favorably channels FA from mitochondria to lipid droplets and support LD biogenesis [28]. PLIN5 overexpression in INS-1 cells promotes LD formation under palmitate overload [204]. Besides its role in FAO, PLIN5 is also known to prevent the interaction between ATGL and CGI58, thereby inhibits lipolysis [205,206]. IFNγ-mediated upregulation of *Plin5* might contribute to LD expansion in the expense of mitochondrial FAO.

In support of this interpretation, respiration studies demonstrated that FA-mediated mitochondrial respiration is impaired in IFNγ-treated cells at 24 h. IFNγ treatment caused a modest decrease in basal respiration, while reducing maximal respiration by 20%, thus also decreased net spare respiratory capacity. Although increased mitochondria FAO is a mechanism to resolve excessive cellular FFAs [207], overload of long chain FFAs has been shown to impair mitochondrial membrane potential and OXPHOS [208-210]. This can be explained by several mechanisms. First, IFNγ-mediated increased DNL can produce malonyl CoA that inhibits CPT1a activity, causing a reduction in mitochondrial FAO. Second, the downregulation of *Pgc1a* can have negative impact on oxidation, as this coactivator is a master regulator of mitochondrial biogenesis and OXPHOS. Increased *Pgc1a* expression enhances mitochondrial biogenesis and OXPHOS in cancer cells [211]. *Pgc1a* deficient mice had hepatic steatosis due to reduced mitochondrial FAO and increased

lipogenic gene expression in the liver [212]. Our data strongly suggest that IFNγ-mediated downregulation of *Pgc1a* contributes to impaired FAO, increased lipogenic gene expression and TAG accumulation in β cells.

Uncoupling proteins UCP promotes proton transport to the matrix, therefore dissociating substrate oxidation from ATP synthesis. While UCP1 is a classic uncoupling protein in adipose tissues and promotes thermogenesis, UCP2 has been argued to play little role in uncoupling [213], but rather is involved in the regulation of FAO [214]. UCP2 is the only member of UCP family expressed in pancreatic β cells and islets [215], and possesses a negative impact on insulin secretion. Our results showed that IFNγ caused a time-dependent upregulation of *Ucp2* from 18 to 24 h. As *Ucp2* expression is induced by FAs [216], it is possible that IFNγ-induced late *Ucp2* expression results from increases intracellular NEFA levels. Another function of UCP2 is the induction of proton leak as it increases the dissipation of protons across the inner membrane [217,218]. UCP2 contributes significantly to proton leak in INS-1 cells [219,220]. As we observed a modest but significant decline in leak respiration in IFNγ-treated cells, there remains a question whether *Ucp2* is involved in cytokine-mediated effects on proton conductance in β cells.

IFNγ-mediated lipid metabolism and β cell secretory function

IFNγ did not affect cellular respiration in response to glucose, suggesting no effect on glucose oxidation. In β cells, insulin secretion is tightly regulated by glucose metabolism; however, FA signaling also plays a significant role in GSIS. While short term exposure to NEFA potentiates GSIS, long term exposure, i.e. 24 h to 48 h, decreases GSIS [221]. Increase TAG synthesis and accumulation also impairs insulin secretion [222-224]. Besides, IFNγ reduced mitochondrial FAO and OXPHOS and upregulated *Ucp2* levels. These factors are known to contribute to defective insulin secretion [225]. In our studies, 24 h treatment with IFNγ trended to decrease basal and glucose-

stimulated insulin secretion in INS-1 cells. It is noteworthy, however, that the insulin secretion was performed in KRBH media without the presence of serum. As IFNγ only reduced FAO but not glucose oxidation, it is possible that IFNγ could impair FA-mediated amplifying effect on GSIS. Nonetheless, it is possible that persisting changes in lipid and transcriptional activation when β cells are exposed to IFNγ chronically in inflammation can pose a harmful effect on β cell secretory function.

IFNγ increases β cell susceptibility to ER stress induced by other proinflammatory cytokines

Although IFNγ-mediated chronic changes in lipid composition enhanced anti-viral function, these lipid species may have detrimental effect on β cell function, i.e. UPR and insulin secretion. Here it was shown that IFNγ did not alter ER stress markers, except for downregulating *sXbp1* levels at 24 h. The absence of robust ER gene activation in this study is supported by previous findings in pancreatic β cells that IFNγ by itself does not cause ER stress but can amplify ER stress caused by cyclopiazonic acid [226] or when used in combination with IL-1β [189,227]. In this study, pretreatment with IFNγ primed INS-1 cells to enhanced UPR in response to other cytokines (IL-1β, TNFα) although IFNγ was absence. The mechanism of this priming effect by IFNγ is still unknown, however, it is possible that IFNγ-mediated increase in TAG and DNL predispose the cells to increase the susceptibility to ER stress upon other stimuli.

In conclusion, the results in this chapter have provided evidence to demonstrate the impact of IFNγ on pancreatic β cell mitochondrial and ER function. Although IFNγ has minimal effects on mitochondrial biogenesis and UPR, long term exposure to IFNγ resulted in declined mitochondrial OXPHOS capacity to FA and accumulation of acyl carnitines. These data suggest that IFNγ reduces FAO while accumulating NEFA and TAG. These metabolic consequences are

marginally detrimental to insulin secretory function, however, possibly prime β cells to cell stress induced by other proinflammatory cytokines.

APPENDIX

Supplemental Table 3-1. PCR primer sequences.

Gene	Protein name	Forward primer (5'-3')	Reverse primer (5'-3')
Cpt1a	Carnitine palmitoyl transferase 1a	AGACCGTGAGGAACTCAAACCCAT	CACAACAATGTGCCTGCTGTCCTT
Cpt2	Carnitine palmitoyl transferase 2	TCCTGCATACCAGCAGATGAACCA	ACAGTGGAGAAACTCTCGGGCATT
Lcad	Long chain Acyl CoA	AATGGGAGAAAGCCGGAGAAGTGA	GAAACCAGGGCCTGTGCAATTTGA
Pgc1a	PPARα coactivator 1α	GACGACAAAGTAGACAAGACCA	CCCAAGGGTAGCTCAGTTTATC
Plin5	Perilipin 5	AACTGAGGGAGAGCAAACAC	TTTGGGTGATGGAAAGTAGGG
Atf4	Activating transcription factor 4	CCATGGCGCTCTTCACGAAAC	GCCAACACTTCGCTGTTCAG
Chop	C/EBP homologous protein	AACTGTTGGCATCACCTCCTGTCT	TCCTCAGCATGTGCACTGGAGATT
sXbp1	Spliced X-box binding protein 1	GAGTCCGCAGCAGGTG	GCGTCAGAATCCATGGGA

Chapter 4. Mechanism of interferon gamma-mediated effect on lipid metabolism gene expression

Abstract

IFNγ was previously shown to alter β cell TAG and FA levels through regulating expression of genes involved in lipolysis, *de novo* lipogenesis and FAO, and play a role in host defense, mitochondrial and ER function of pancreatic β cells. The signaling pathways whereby IFNγ transcriptionally regulates these genes are not known. In this chapter, it was demonstrated that IFNγ regulates FA and TAG metabolism in a distinctively dynamic manner compared to other inflammatory cytokines. Gene expression and LD staining suggested that IL-6 induces lipolysis; while a mixture of TNFα and IL-1β enhanced lipogenesis. type 1 IFN (IFNα) showed a delayed upregulation of *Pnpla2*, *Plin1/2* and *Cpt1a* compared to IFNγ, and upregulated *Dgat2* at 24 h. These results suggest that long term exposure to IFNγ and pro-inflammatory cytokines TNFα, IL-1β and type 1 IFN, collectively result in TAG accumulation in pancreatic β cell via different mechanisms. Next, it was determined whether IFNγ transcriptionally regulates lipid metabolism through its actions on Janus kinase (JAK)-Signal Transducer and Activator of Transcirption (STAT) activation. Preincubation of INS-1 cells with JAK1/2 inhibitor ruxolitinib completely abolished IFNγ-mediated regulation of lipid genes, suggesting that the transcriptional effects of IFNγ are JAK1/2-dependent. STAT3, a member of the STAT family shown to regulate lipid metabolism in adipose tissue and liver, was tested for its involvement in IFNγ-mediated effect in β cell. Phosphorylation and activation of STAT3 was inhibited by siRNA or the pharmacological inhibitor of STAT3 nifuroxazide. Neither approaches blocked IFNγ-mediated gene expression at 24 h, suggesting that STAT3 is not involved in the late effect of IFNγ on gene transcription. Nifuroxazide, however, abolished IFNγ-mediated downregulation of *G0s2* at 6 h, indicating that

STAT3 mediates IFNγ-induced transient lipolysis. Unexpectedly, nifuroxazide, a JAK2/TYK2 inhibitor, upregulated basal levels of *Pnpla2*, *Cpt1a* and *Plin1* to the same extent as IFNγ, suggesting a possible feedback mechanism among tyrosine kinases and downstream STATs to regulate constitutive and IFNγ-induced expression of metabolic genes. In summary, we demonstrated that IFNγ and other inflammatory cytokines have specific impact on transcriptional expression of genes related to lipolysis, LD proteins and TAG synthesis in β cells. IFNγ-mediated transcriptional regulation of lipid metabolism is JAK1/2-dependent, however, it is possible that multiple JAKs/STATs are involved in different phases of its dynamic regulation.

4.1. Introduction

Many cytokines and growth factors exert their biological effects through the activation of Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathways. Cytokines bind to their receptors, which are bound constitutively with tyrosine kinase JAKs, causing receptor conformational change and trans-phosphorylation of JAKs. Activated JAKs recruit STAT proteins to the receptor and phosphorylate them, mainly at conserved tyrosine residues near the C-terminus [228]. Phosphorylated STATs form hetero- or homodimers and enter the nucleus to initiate transcription of genes that regulate inflammatory responses, immune function, and cell proliferation [229]. In mammals, there are four members of JAK (JAK1/2/3, TYK2), and six members of STAT (STAT1/2/3/4/5a/5b/6). Unique subsets of JAKs bind to the cytoplasmic region of each cytokine receptor; however they can activate a variety of STATs, rendering the complexity and versatility of cytokine-induced effects. For example, IFNγ activates JAK1/2 and STAT1/3/5, while type 1 IFN activates JAK2/TYK2 and STAT1/2/3/4/5. IL-6 activates STAT3 through JAK1/2/TYK2 [228]. Although cytokines signal through similar downstream STAT, they have their own specificity apart from their overlapping effects on the

transcriptome [230].

Besides well-known function in immunity, some members of the STAT family have been shown to regulate lipid metabolism in highly metabolic tissues [231,232]. Particularly, STAT3 is known to play a central role in lipid metabolism in adipose tissue, liver, and skeletal muscle. Studies of STAT3 overexpression/deletion or activation of STAT3 by IL-6 have shown that it stimulates lipolysis in adipocytes [233] and inhibits lipogenesis in hepatocytes [234,235]. Besides STAT3, the role of other STATs on regulation of lipid metabolism is underexamined. STAT1 has been shown to bind to PPARδ promoter [236], downregulate *Lpl* expression in adipocytes [41,237], and involved in mitochondrial oxidation of tumor cells [238]. Lack of STAT5 induced hepatic steatosis through elevating *Cd36, PPARδ, Pgc1α/β* and *Fasn* expression [239]. In pancreatic islets and β cells, the contribution of STATs is solely recognized in inflammation and cellular dysfunction. TNFα and IL-1β in combination with IFNγ signal through STAT1 to induce β cell apoptosis [118]. Hyperglycemia and hyperlipidemia together cause β cell death via activation of STAT1 and NF-κb [240]. The roles of JAK/STAT in islet and β cell lipid metabolism and mitochondrial function remain unexplored.

IFNγ was previously shown to dynamically regulate gene transcription of key enzymes and proteins involved in lipid metabolism pathways in β cells. Herein, we investigated the signaling pathway responsible for IFNγ-mediated effects. First, the specific transcriptional effects of IFNγ on key enzymes regulating FA and TAG metabolism were compared with type 1 IFN, IL-6, and a mixture of IL-1β and TNFα. Next, it was examined whether IFNγ exerts its transcriptional effects via the classical JAK/STAT pathway, and whether STAT3 is involved in the regulation of lipid metabolism in β cells. Understanding the signaling pathway whereby IFNγ regulates β cell lipid metabolism is crucial to identify therapeutic targets to modify the impacts of cytokines for the

prevention and treatment of T1D.

4.2. Materials and methods

Cell culture. INS-1 cells (passage 71-84) were cultured in RPMI 1640 media containing 11.1 mM glucose, supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, and 10 mM HEPES (INS-1 media) at 37^{o}C in a humidified incubator containing 95% air and 5% CO2. In all experiments, cells were seeded at a density of 0.25x10^{6} cells/cm^{2}. Twenty-four hours after plating, cells were washed and incubated for an additional 24 h in INS-1 media containing 10% heat-inactivated FBS (hiFBS media). Cells were treated with 100 U/ml rat recombinant IFNα, 50 ng/ml rat recombinant IFNγ, 100 ng/ml rat recombinant IL-6, or a mixture of 120 pg/ml human recombinant IL-1β and 25 pg/ml human recombinant TNFα (R&D system), or vehicle control (PBS containing 0.0001% BSA) for indicated lengths of time. Cells were then washed and harvested for mRNA analysis.

siRNA transfection. INS-1 cells (2x10^{6} cells) were resuspended in electroporation buffer (0.14 mM ATP-disodium salt, 0.23 mM MgCl2, 66.7 mM K2HPO4, 13.7 mM NaHCO3, 2.16 mM glucose, pH 7.4) and added to 1mm gap electroporation cuvette with siSTAT3 (Dharmacon siGenome Smart pool) or mock at final concentration of 100nM siRNA. Transfection was performed with Amexa Transfection system using program D-026. Electroporated cells were added to individual wells of 6-well plate and sub-cultured in INS-1 media for 24 h. Cells were then washed and cultured in hiFBS media for an additional 24 h. Cells were incubated with IFNγ for indicated lengths of time, and harvested for mRNA and protein expression.

JAK inhibitors. JAK1/2 inhibitor ruxolitinib (Selleck Chemical LLC) was kindly provided by Dr. Das, Michigan State University) at 200 mM stock concentration. JAK2/TYK2/STAT3 inhibitor Nifuroxazide (MiliporeSigma) was dissolved in DMSO to gain a stock concentration of

100 mM. Cells were sub-cultured for 48 h as described above, then treated with JAK inhibitors at 10 μM final concentration for 2 h. After that, cells were treated with IFNγ for 30 mins, 6 h or 24 h. Cells were harvested for protein and mRNA expression.

cDNA synthesis and real time PCR. Total RNA was extracted with Trizol (Invitrogen, CA). RNA was converted into cDNA using High Capacity cDNA synthesis kit (Applied Biosystems). cDNA template was amplified for qPCR using SYBR green and detected by 7500 qPCR amplification system (Applied Biosystems). Gene expression is calculated as fold change relative to cyclophilin mRNA levels and compared to control samples using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting. Total protein lysates were obtained by lysing cells in RIPA lysis buffer with protease inhibitor cocktails (Sigma). Protein levels were measured by Lowry assay and equal amounts of protein (30-40 ug) was resolved on a gradient (4-20%) or 10% SDS-PAGE. Proteins on gels were transferred to a PVDF membrane (Millipore, MA). Membranes were blocked in 7% milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated with primary antibodies overnight. Primary antibodies used: rabbit monoclonal anti-Y701 p-STAT1, rabbit monoclonal anti-Y705 p-STAT3, rabbit monoclonal anti-STAT3 (Cell Signaling), goat anti-actin (Santa Cruz), mouse anti-tubulin (Sigma) at 1:1,000 dilution in 7% milk/TBS-T. The membranes were washed with TBS-T and incubated with secondary antibodies for 1 h at room temperature. Secondary antibodies used were Alexa Fluor® 680 goat anti-rabbit IgG (Invitrogen, CA), IRDye 800CW donkey anti-goat (LI-COR Biosciences, NE) at 1:10,000 dilution. Membranes were washed and imaged using LICOR system. Immunoreactivity was quantified by Image Studio Lite (version 4.0) software. Actin or tubulin were used as loading control.

Statistical analysis. Data analysis were performed by GraphPad Prism (version 7.0). Data are shown as the mean± SEM. Comparisons between groups were performed by unpaired

Students' t tests and corrected for multiple comparisons by Bonferroni method, or two-way ANOVA with Bonferroni's correction for multiple comparison, as specified in the figure legends. $p < 0.05$ was considered statistically significant.

4.3. Results

4.3.1. The specific effects of IFNγ on metabolic gene expression and LD formation

Treatment of INS-1 cells with IFNγ has been shown to temporally regulate genes coding for enzymes and regulators of lipolysis and LD metabolism, thus causing a biphasic change in levels of TAG (Chapter 2). IL-6 and TNFα have been shown to induce lipolysis in adipocytes or enhance lipogenesis in hepatocytes [2] (**Table 1-1 and Table 1-2**). The biphasic effects of IFNγ prompted us to investigate the differential impact of IFNγ and other inflammatory cytokines on expression of genes regulating TAG metabolism. INS-1 cells were exposed to IFNγ or IL-6, or a mixture of IL-1β and TNFα for 12 and 24 h. Unlike the biphasic regulation observed for IFNγ, IL-6 downregulated *G0s2* at both timepoints, while modestly upregulating *Pnpla2* at 12 h (**Fig. 4-1*A***), indicating sustained lipolysis. Neither IL-6 nor TNFα+IL-1β altered *Plin1/2/5*, or *Accα* levels, while IFNγ upregulated these genes at both time points (**Fig.4-1*B***). TNFα and IL-1β although did not alter these lipolytic genes but significantly upregulated *Dgat2* mRNA level at 24 h (**Fig. 4-1*C***). Both IL-6 and IFNγ downregulated *Pgc1a*, but the effect of IL-6 was seen earlier at 12 h (**Fig.4-1*D***). These data suggest that the impact of IFNγ on transcriptional regulation of lipid metabolism is uniquely dynamic and involve many target genes of different pathways.

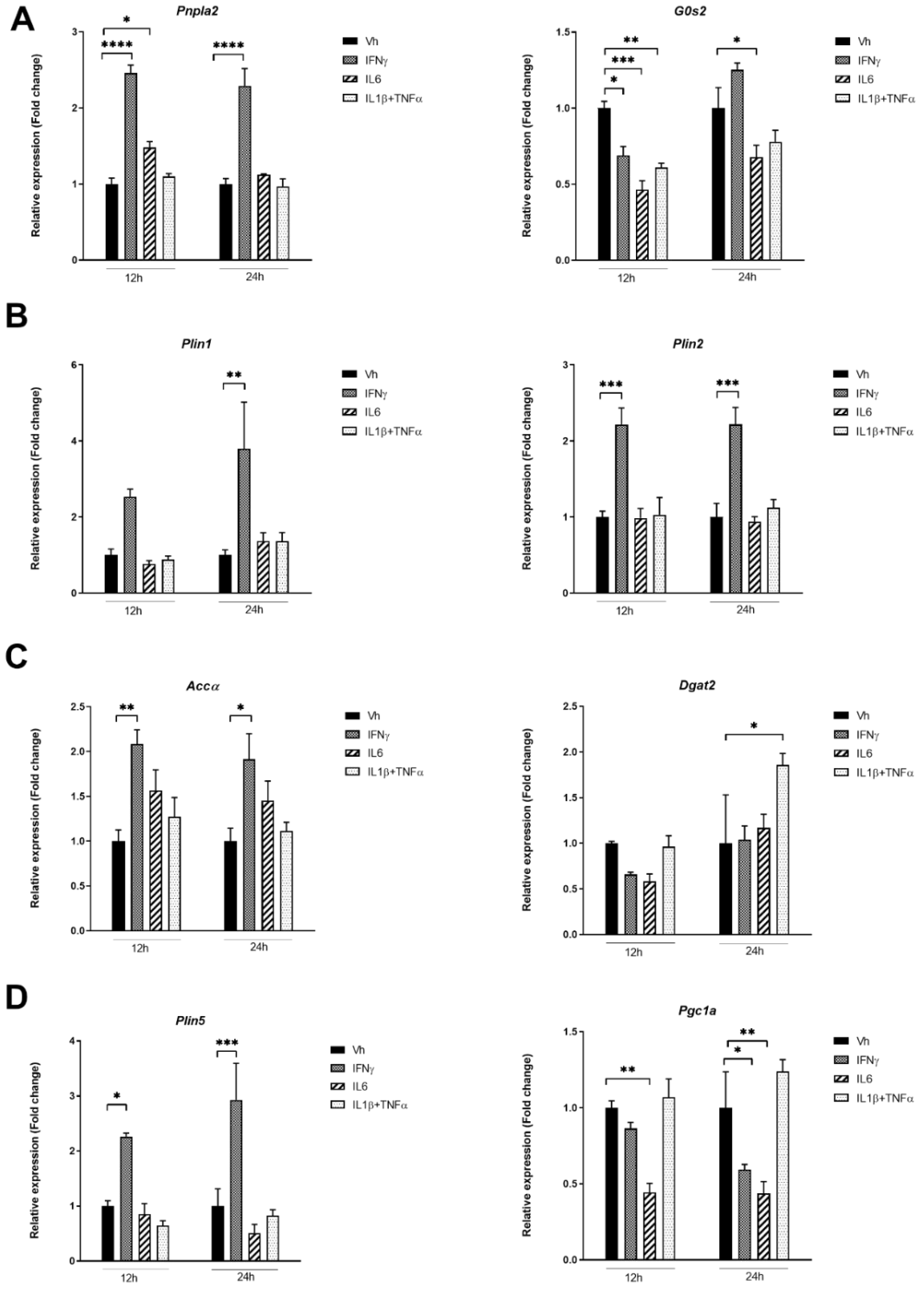


Figure 4-1. Differential transcriptional regulation of IFNγ and other proinflammatory cytokines on genes involved in FA and TAG metabolism.

INS-1 cells were treated with 50 ng/ml IFNγ, 100 ng/ml IL-6, or a mixture of 120 pg/ml IL-1β and 25 pg/ml TNFα for 12 and 24 h. mRNA levels of genes regulating (**A**) lipolysis, (**B**) lipogenesis,

Figure 4-1 (cont'd)
(**C**) LD biogenesis and (**D**) FAO were measured. Data are mean ± SEM (n = 3), shown as fold change to control at each timepoint. *p < 0.05, **p < 0.01, ***p < 0.001 by multiple t-tests with Bonferroni correction.

The gene expression results also suggest that proinflammatory cytokines have distinctive effects on β cell intracellular TAG metabolism, especially after long term (24 h) exposure. To investigate the impact of IFNγ and other cytokines on LDs formation in INS-1 cells, cells were exposed to these cytokines for 24 h, after which LDs were visualized by Oil Red O staining. As previously reported, IFNγ increased formation of cluster of LDs that mainly localized around the perinuclear region. In contrast, treatment with IL-6 led to decreased numbers of LDs and these droplets are much smaller. On the other hand, TNFα+IL-1β caused significant elevation of LD number in INS-1 cells, and these LDs are unilocular and larger than those of IFNγ-treated cells and found in cytoplasm instead of perinuclear region (**Fig. 4-2**).

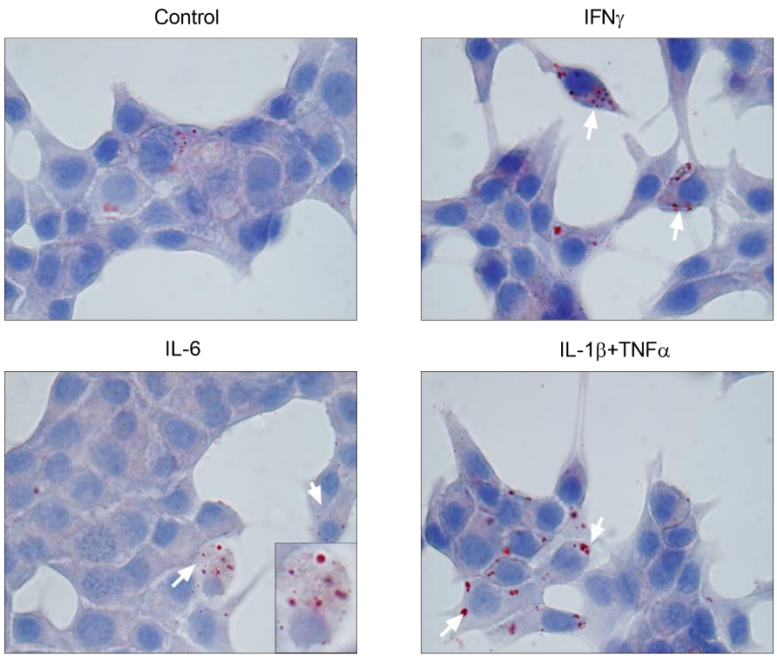


Figure 4-2. Quantity and localization of lipid droplets under exposure to different cytokines. INS-1 cells were treated with IFNγ, IL-6, or mixture of IL-1β+TNFα for 24 h. LDs were visualized by Oil Red O staining. Images are representative of at least 10 frames per sample and from two

Figure 4-2 (cont'd)
independent experiments. Arrows are pointed at LDs. Images were taken at 100X magnification.

4.3.2. Different kinetics between interferons in regulating genes involved in TAG metabolism

In our LEW.1WR1 rat studies, we observed a time-dependent regulation among type 1 and type 2 IFN-induced gene expression, i.e. a sustained upregulation of IFNγ-induced gene *Ubd* compared to the rapid restoration to basal of *Irf7*, an type 1 IFN-induced gene. This has prompted us to hypothesize that IFNγ signaling is more stable than type 1 IFN and may play a role in the change of islet TAG levels. In INS-1 cells, time-course studies showed that IFNγ-treated cells have elevated IFNα expression from 6 h to 24 h, whereas IFNβ levels were only upregulated from 12 h (**Supp. Fig. 4-1**). This raises the possibility that type 1 IFNs might modulate IFNγ's effect on regulation of lipid metabolism genes through an autocrine action. To investigate this possibility, the impact of IFNα, a member of type 1 IFNs on gene expression involved in TAG metabolism was examined. Treatment of INS-1 cells with IFNα led to a rapid (30 min) increase of tyrosine phosphorylation of STAT1 (at the tyrosine Y701 residue), but did not affect STAT3 phosphorylation (**Fig. 4-3*A***). Next, the temporal effects of IFNα and IFNγ on expression of interferon stimulated genes (ISGs) and genes regulating TAG metabolism were analyzed. IFNγ, but not IFNα strongly upregulated *Ubd* at both 12 h and 24 h. There was a sustained activation of anti-viral genes *Mx1* and *Irf7* by IFNγ at 12 and 24 h, however IFNα-mediated upregulation of *Mx1* and *Irf7* were markedly reduced at 24 h (**Fig. 4-3*B***). In terms of genes regulating TAG metabolism, IFNα showed a delayed effect compared to IFNγ, as it only upregulated *Pnpla2, Cpt1a* and *Plin1* levels at 24 h (**Fig. 4-3*C***). Importantly, IFNα had no effect on *G0s2* and strongly upregulated *Dgat2* at 24 h, while IFNγ exerted its biphasic regulation on *G0s2* and downregulated *Dgat2* as previously shown in chapter 2 (**Fig. 4-3*C***). These data demonstrate different kinetics of

IFNγ and IFNα in the transcriptional activation of not only ISGs but also genes mediating TAG metabolism, suggesting that IFNs regulate lipid metabolism through their specific but overlapping signaling pathway.

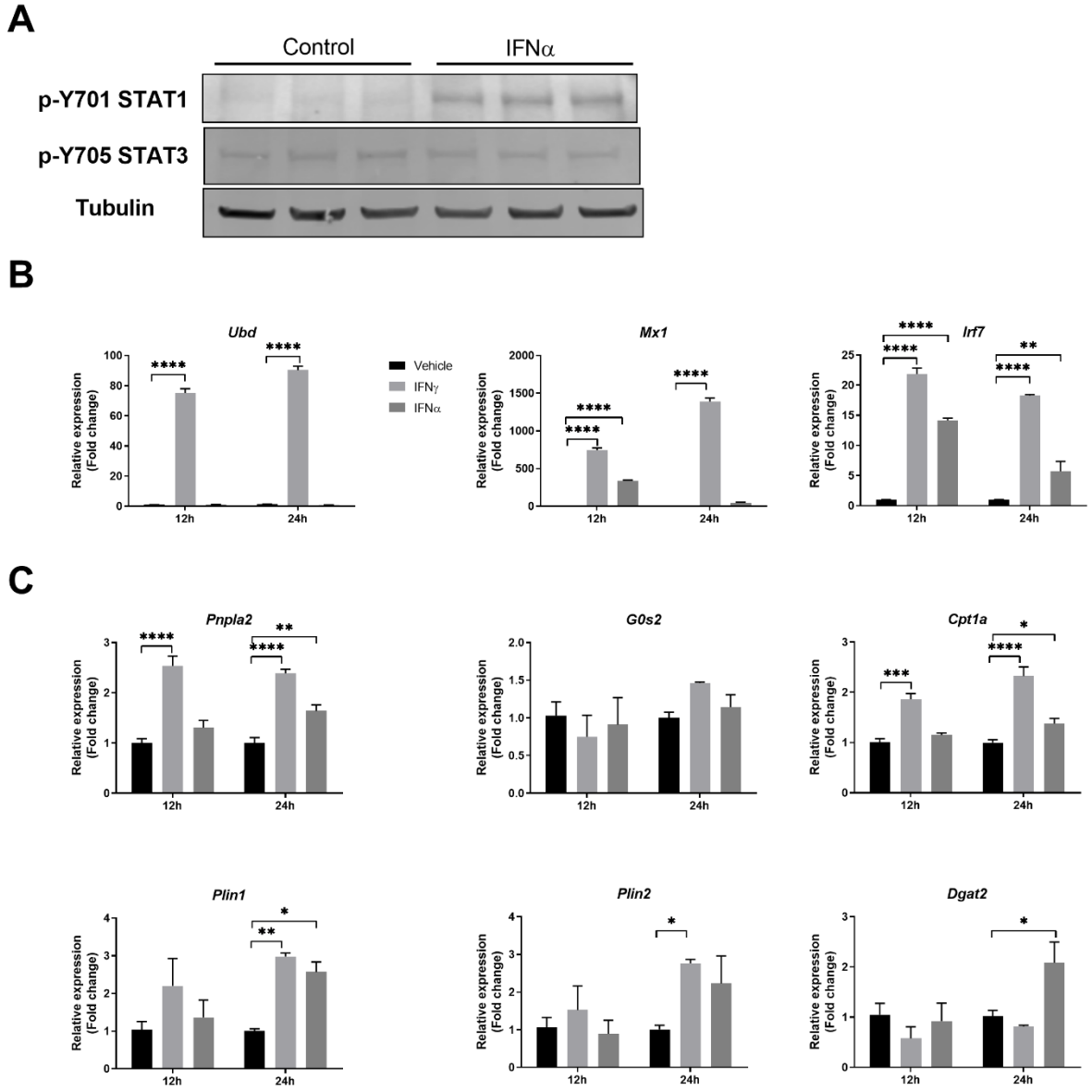


Figure 4-3. **Different temporal effects of IFNα and IFNγ in regulation of genes involved in TAG metabolism.**
(A) Tyrosine phosphorylation of STAT1 and STAT3 in INS-1 cells treated with 100 U/ml IFNα for 30 mins. Blot is the representative of three different wells of one experiment. (**B-C**) INS-1 cells were treated with 50 ng/ml IFNγ or 100 U/ml IFNα for 12 and 24 h. mRNA levels of ISGs **(B)** and genes regulating TAG and LD metabolism **(C)** were measured. Data are mean ± SEM (*n* = 3),

Figure 4-3 (cont'd)
shown as fold change to non-treated cells (control) at each timepoint. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$ by multiple t-tests with Bonferroni correction.

4.3.3. The contribution of JAKs in IFNγ-mediated transcriptional activity

IFNγ activates its classical signaling pathway through the IFNGR receptor, which leads to the autophosphorylation of constitutively bound JAK1/2. Therefore, it was examined whether JAK1/2 is involved in IFNγ-mediated gene transcription of lipid metabolism pathways. INS-1 cells were pretreated with Ruxolitinib, an FDA-approved JAK1/2 inhibitor for 2 h before treatment with IFNγ. Ruxolitinib completely inhibited IFNγ-induced phosphorylation of STAT1, validating its blockade of JAK1/2 kinases (**Fig. 4-4*A***). Gene expression results at 6 h and 24 h showed that pretreatment with ruxolitinib abrogated IFNγ-mediated changes in *G0s2*, *Pnpla2*, *Plin2/5*, *Acca*, and *Pgc1a* (**Fig. 4-4*B***). Notably, ruxolitinib treatment by itself strongly enhanced basal level of *Pgc1a* in control cells (**Fig. 4-4*B***). These data indicate that IFNγ-mediated transcriptional regulation of genes involved in TAG and FA metabolism is JAK1/2-dependent.

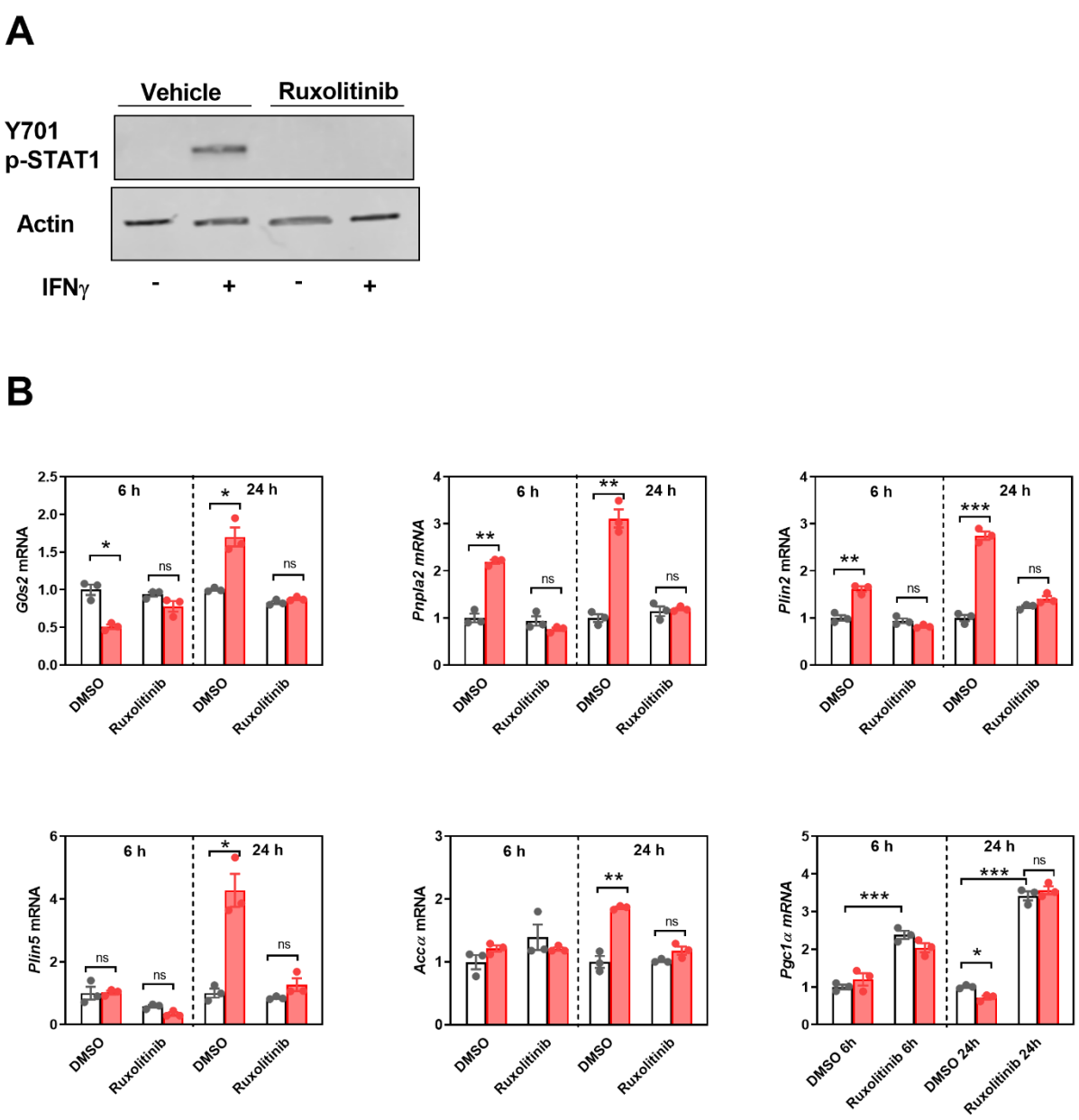


Figure 4-4. IFNγ-mediated regulation of lipid gene expression is JAK1/2 dependent. INS-1 cells were pretreated with 10 μM ruxolitinib**,** then treated with IFNγ for 30 mins **(A)** or 6 h and 24 h (B). (A) Levels of tyrosine-phosphorylated STAT1 were measured with immunoblotting. Blot is the representative of three different wells of one experiment. (**B**) mRNA levels of genes regulating TAG and FA metabolism were measured. Data are mean ± SEM (n = 3), shown as fold change relative to non-treated cells (control) at each timepoint. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001 by two-way ANOVA with Bonferroni correction for multiple comparison.

4.3.4. The role of STAT3 in IFNγ-induced transcriptional activation

STAT3 has been shown to regulate lipid metabolism induced by adipokines and cytokines,

especially leptin and IL-6. To investigate whether STAT3 is involved in IFNγ-mediated gene transcription, INS-1 cells were transfected with siRNA against STAT3. Gene expression results validated that siSTAT3 reduced basal *Stat3* mRNA by 70% ($p = 0.09$), and significantly lowered IFNγ-induced *Stat3* expression by 60% ($p < 0.0001$) (**Fig. 4-5*A***). Decreased total STAT3 protein and IFNγ-activated tyrosine/serine phosphorylation of STAT3 were also confirmed by immunoblotting (**Fig. 4-5*B***). siSTAT3, however, did not alter IFNγ-mediated upregulation of *Pnpla2*, *Cpt1a*, or downregulation of *Dgat2* at 24 h. siSTAT3 also tended to block IFNγ-induced *G0s2* upregulation but the difference did not reach significance ($p = 0.06$) (**Fig. 4-5*C***).

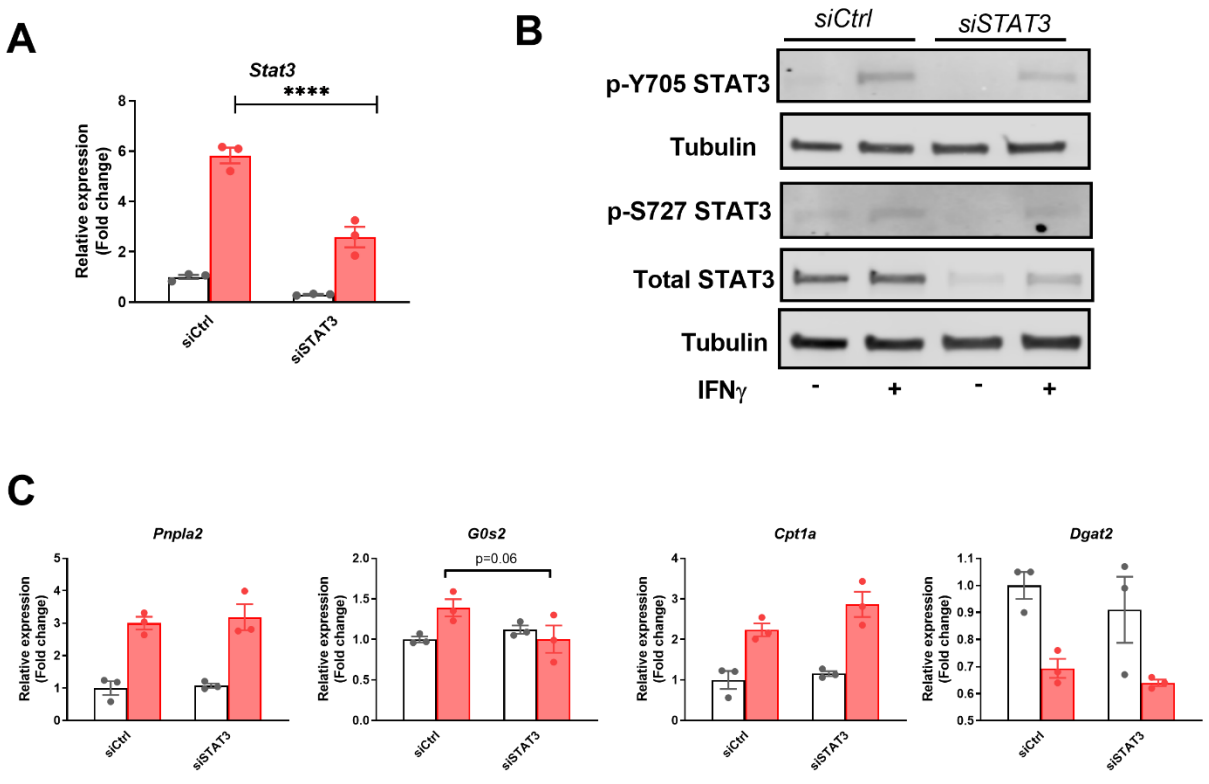


Figure 4-5. STAT3 is not involved in the late effects of IFNγ in regulation of lipid metabolism genes.
(**A-C**) INS-1 cells were transfected with 100 μM siSTAT3 or mock control and cultured for 48 h, then treated with IFNγ for 24 h (**A** and **C**) or 30 mins (**B**). mRNA expression of *Stat3* (**A**) and genes regulating lipid metabolism altered by IFNγ (**C**) was measured by qPCR. Data are mean ± SEM ($n = 3$), shown as fold change relative to non-treated cells (control). **** $p < 0.0001$ by two-way ANOVA with Bonferroni correction for multiple comparison. (**B**) Levels of tyrosine, serine

Figure 4-5 (cont'd)
phosphorylated STAT3 and total STAT3 were measured by immunoblotting. Blots are representative of two technical replicates in one experiment.

Nifuroxazide, a pharmacological inhibitor of STAT3, JAK2 and TYK2 [241] was also tested for the effects of STAT3 on expression of lipid metabolism genes. Pretreatment with 10 μM nifuroxazide reduced IFNγ-induced STAT3 as well as STAT1 phosphorylation (**Fig. 4-6*B***), although slightly increased IFNγ-induced *Stat3* mRNA (**Fig. 4-6*A***). Unlike ruxolitinib, nifuroxazide treatment did not attenuate IFNγ-induced *Pnpla2*, *Cpt1a* and *Plin1* expression (**Fig. 4-6*C***) at 24 h. Unexpectedly, nifuroxazide upregulated these genes in control cells, and had additive effects on gene expression when combined with IFNγ (**Fig. 4-6*C***). Nifuroxazide caused similar potentiating effects on IFNγ-induced *Cxcl10* expression, but not *Oas1* (**Fig. 4-6A**). In contrast to the 24 h results, nifuroxazide pretreatment blocked IFNγ-mediated downregulation of *G0s2* at 6 h (**Fig. 4-7**), suggesting that G0s2 suppression is mediated through JAK2/TYK2/STAT3 signaling. Nifuroxazide also significantly downregulated *G0s2* levels in control cells. Nifuroxazide did not alter IFNγ-induced expression of *Pnpla2*, *Cpt1a* or *Plin1* at 6 h (**Fig. 4-7**), and slightly enhanced their basal levels. Together these data suggest that STAT3 is likely responsible for IFNγ-mediated downregulation of *G0s2* and activation of transient lipolysis, whereas other STAT proteins are involved in the upregulation of other lipolytic and LD genes.

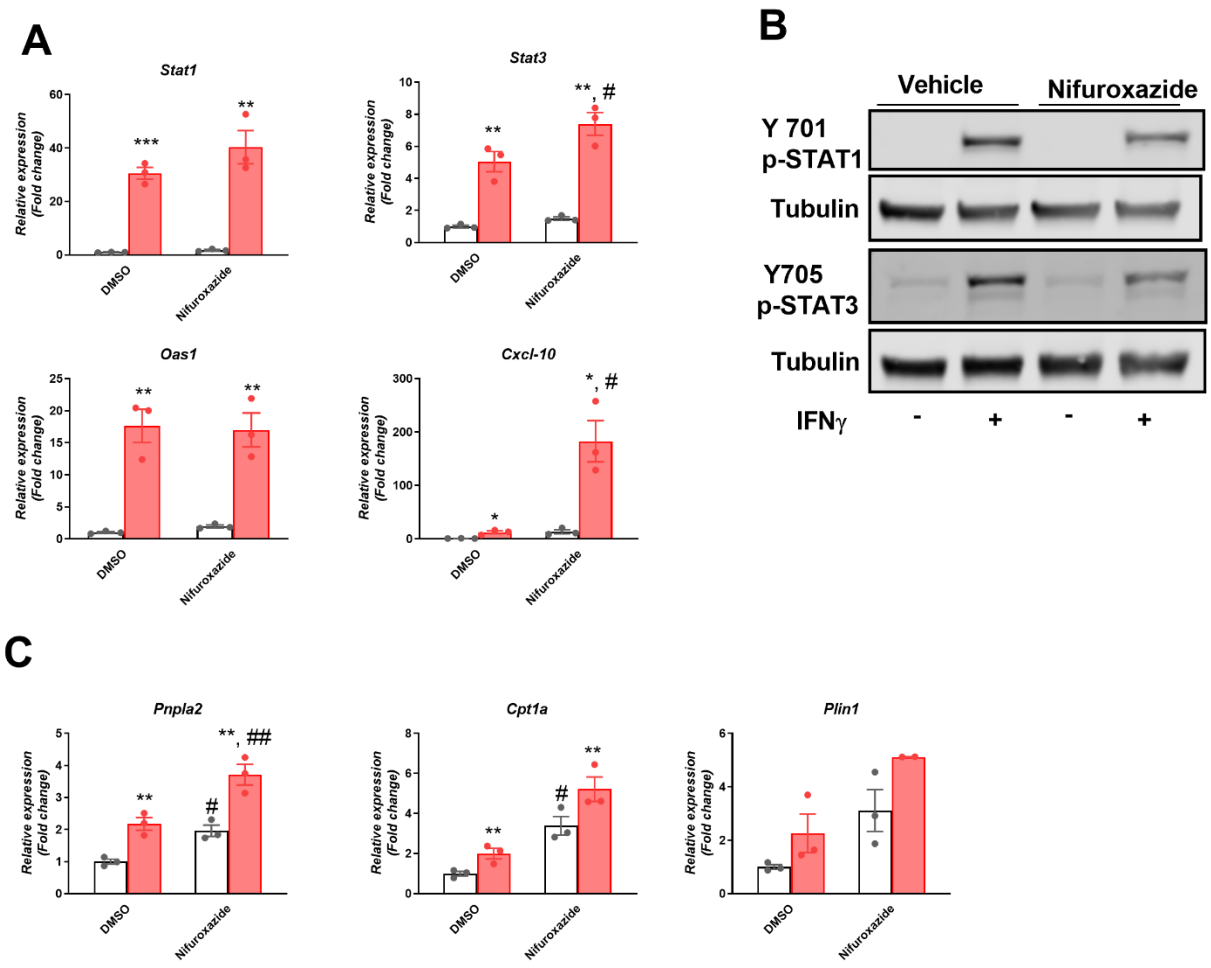


Figure 4-6. JAK/STAT plays a role in the constitutive expression of genes regulating lipid metabolism.

(A-C) INS-1 cells were pretreated with 10 μM nifuroxazide for 2 h, then treated with IFNγ for 24 h (A and C) or 30 mins (B). mRNA expressions of ISGs *Stat1*, *Stat3*, *Oas1* and *Cxcl-10* **(A)** and genes regulating lipolysis and LD metabolism **(C)** were measured. Data are mean ± SEM (n = 3), shown as fold change to non-treated cells (control). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between IFNγ-treated cells and vehicle control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ between nifuroxazide and DMSO-pretreated cells, by two-way ANOVA with Bonferroni correction for multiple correction. Red bar: IFNγ, white bar: control. **(B)** Protein levels of tyrosine phosphorylated STAT1/3 were measured. Blots are representative of two independent experiments. Tubulin was used as loading controls.

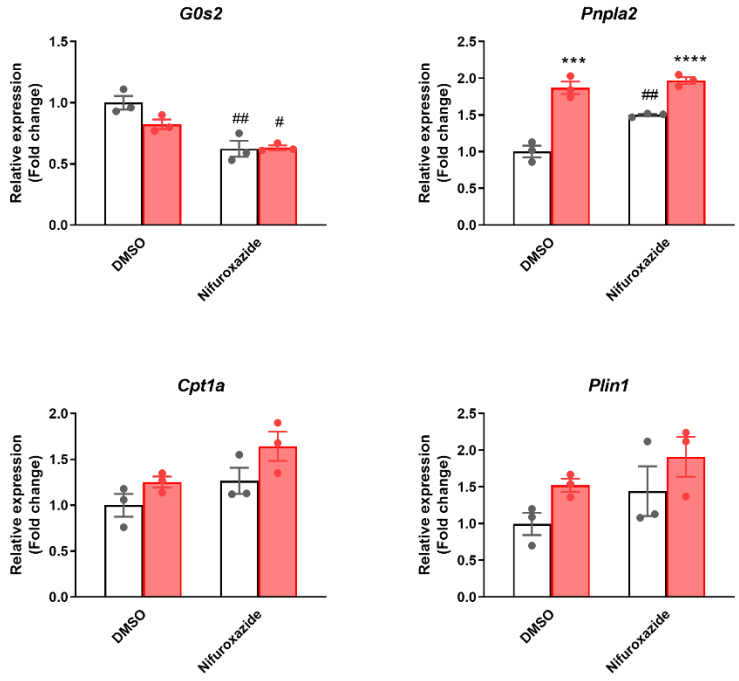


Figure 4-7. STAT3 mediates IFNγ-induced early lipolysis.
INS-1 cells were pretreated with 10 μM nifuroxazide for 2h, then treated with IFNγ for 6 h. mRNA expressions of genes regulated by IFNγ were measured. Data are mean ± SEM (n = 3), shown as fold change to non-treated cells (DMSO+control). ***$p < 0.001$ between IFNγ-treated cells and vehicle control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ between nifuroxazide and DMSO-pretreated cells, by two-way ANOVA with Bonferroni correction for multiple correction. Red bar: IFNγ, white bar: control.

4.4. Discussion

IFNγ was previously shown to regulate mRNA expression of genes participating in FA and TAG metabolism, leading to dynamic changes in levels of TAG, NEFA and LD in β cells. Herein we demonstrated that the gene expression pattern by IFNγ is uniquely dynamic compared to type 1 IFN and other inflammatory cytokines. It was further shown that IFNγ exerts its transcriptional effect via its receptor-bound JAK1/2, which possibly activate multiple downstream STATs and govern different phases of IFNγ-mediated effects.

IFNγ regulates gene expression in a distinctly different manner from type 1 IFN and other inflammatory cytokines

In this study, we have shown that IFNγ is unique in its regulation of TAG and LD levels. IFNγ upregulates *Pnpla2* and regulated *G0s2* levels in a biphasic manner, indicating transient lipolysis and followed by suppression of lipolysis. In contrast, IL-6 sustainably downregulated *G0s2,* indicating enhanced lipolysis. Consistent with this, IL-6 treated cells had significant less LDs, and the LDs were much smaller. In agreement with our observation, IL-6 induces lipolysis in adipose tissue and skeletal muscle [242-244]. Whereas IL-6-induced lipolytic effects are generally conserved among cell types, the effects of TNFα+IL-1β on lipid metabolism appear to be more cell type dependent. TNFα and IL-1β, individually or in combination, promotes lipolysis in adipocytes, while stimulating lipogenesis in hepatocytes (**Table 1-1** and **1-2**). In INS-1 cells, TNFα+IL-1β treatment upregulated *Dgat2* and resulted in formation of large LDs in the cytoplasm, suggesting enhanced lipogenesis. Interestingly, the distribution of LDs is visually different from those in IFNγ-treated cells. IFNγ treatment led to formation of clusters of small to medium LDs, typically around perinuclear region. On the other hand, TNFα+IL-1β caused the formation of large unilocular cytoplasmic LDs. The central theory for LD biogenesis is that LDs are formed from ER membrane upon *de novo* synthesis of neutral lipids between the bilayers of ER. Other studies, however, have suggested LD growth can occur in the cytosol by *in situ* synthesis via synthetic enzymes present on LD surface [245]. LD can increase size by fusion of small LDs via action of CIDE proteins [246,247]. The mechanisms that dictate the difference among cytokine-induced LDs population were not explored, but they can involve many pathways from synthesis to turnover of LDs. Collectively, these results suggest that proinflammatory cytokines IFNγ, IL-1β and TNFα cause TAG accumulation in INS-1 β cells via different mechanisms and may contribute to cytokine-induce ER stress and insulin secretory defects in the progression towards β cell dysfunction. A previous report, however, suggested that 24 h exposure to a mixture of three

cytokines at sublethal concentration caused enhanced phosphorylation of ACCα and decreased cellular TAG levels in BRIN-BD11 β cells, suggesting reduced lipogenesis and increased FAO [248]. We argue that this difference can arise from the concentrations of cytokines. Low levels of cytokines used in that study can possibly stimulate FAO to provide energy needed for initiation of transcription. However, long term exposure to high dose of proinflammatory cytokines is likely to cause TAG accumulation in β cells, which can contribute to disrupted ER integrity and cell death.

Between two types of IFNs, we observed different kinetics in the regulation of *Pnpla2, Cpt1a* and *Plin1/2*. IFNα only upregulated those genes at 24 h, whereas IFNγ led to early and sustained upregulation. This is contrast with the pattern of expressions of anti-viral genes *Mx1* and *Irf7*, where IFNα response quickly faded after 12 h. Similar kinetics of *Mx1* and *Irf7* were observed in islets of LEW1.WR1 rats injected with PIC (Chapter 2). The classical signaling of type I IFNs involve the formation of IFN-stimulated gene factor 3 (ISGF3) complex, which consists of phosphorylated STAT1 and STAT2 and unphosphorylated interferon regulatory (IRF-9). ISGF3 translocates to the nucleus and binds to IFN-stimulated response element (ISREs) present in the promoters of many ISGs. Substantial evidence has demonstrated a non-canonical role of unphosphorylated STAT1 (U-STAT1) in the temporal regulation of ISGs by two types of IFNs. While the increase in phosphorylated STAT1 only last for a few hours after IFN stimulation, increase in U-STAT1 is suggested to be responsible for the amplification or sustainment of IFNγ-induced gene activation [249]. Recent study showed that IFNα only upregulates U-STAT1 beyond 8 h simultaneously with the decrease of phosphorylated STAT1 levels in hepatic carcinoma cell line [250]. In INS-1 cells, there was a significant increase in mRNA levels of *Stat1* at 12 and 24 h by IFNγ but not IFNα. Therefore, it is likely that IFNγ induces U-STAT1 levels at a faster and more substantial level compared to IFNα, leading early and sustained upregulation of genes involved in

lipid metabolism.

The role of JAK/STAT in IFNγ-mediated transcriptional regulation of lipid metabolism

Our data demonstrate that IFNγ and other cytokines share certain target genes which regulate lipolysis and lipogenesis, yet the pattern of regulation is unique for each cytokine. The canonical effect of IFNγ is through the activation of IFNGR-bound tyrosine kinases JAK1 and JAK2, which phosphorylate STAT1 at the tyrosine 701 residue and activate STAT1 transcriptional activity. JAK-independent pathways, for example NF-κB and PI3K/Akt, are also involved in IFNγ-mediated non-canonical effects [251,252]. Here we demonstrated that pretreatment with ruxolitinib completely attenuated IFNγ-mediated transcriptional regulation of lipid metabolism genes at both 6 h and 24 h, suggest that IFNγ alter lipid metabolism is JAK1/2 dependent.

The contribution of STATs to IFNγ-mediated transcriptional regulation of lipid metabolism is likely much more complex. STAT3 has been shown to play a central role in lipid metabolism in adipocytes and hepatocytes, and previous ChIP seq data demonstrated STAT3 binding sites on many genes regulating lipid metabolism including *G0s2*, *Pnpla2*, *Cpt1a* and *Plin1* [253,254]. In INS-1 cells, however, inhibition of phosphorylated STAT3 by knockdown with siRNA or pharmacological inhibition did not attenuate IFNγ-induced gene transcription at 6 h and 24 h, suggesting that STAT3 does not play a central role in IFNγ metabolic effects in INS-1 β cells. Nonetheless, siSTAT3 slightly attenuated IFNγ-induced *G0s2* at 24 h, and nifuroxazide restored the downregulation of *G0s2* by IFNγ at 6 h, suggesting that STAT3 is responsible for the IFNγ-mediated transient lipolysis. The sustained downregulation of *G0s2* by IL-6 and its associated lipolysis support this interpretation. A previous report also showed that IFNγ transiently activates STAT3 while having a long lasting STAT1 activation in adipocytes [47]. In this study, IFNγ-mediated downregulation of lipogenesis and TAG storage was suggested to be dependent upon

STAT1, since activation of STAT3 by leptin did not reproduce the effects of IFNγ. It is noteworthy that changes in TAG level in IFNγ-treated adipocytes also showed a biphasic response with an insignificant increase at 4 h, followed by significant decreases at 24 h and 48 h. Our data in INS-1 cells indicate that the temporal effects of IFNγ on STAT3 can occur in pancreatic β cells, however, the overall effects of STAT3 activation on TAG levels are cell type dependent.

The role of JAK/STAT in constitutive expression of genes involved in lipid metabolism

The results with JAK inhibitors nifuroxazide and ruxolitinib also reveal a complex role of JAK/STAT proteins in the constitutive expression of lipid metabolism genes. Inhibition of JAK2/TYK2 by nifuroxazide led to elevated basal levels of *Cxcl10*, *Pnpla2*, *Cpt1a*, *Plin1*, and decreased *G0s2* levels. It has been shown that constitutively expression of many genes is regulated by U-STAT [255]. *Cxcl10* levels were shown to be correlated with *Stat1* mRNA level, not tyrosine phosphorylated STAT1 [256]. U-STAT1 has been shown to be negatively regulated by STAT2, as U-STAT2 competitively binds to U-STAT1 and prevents U-STAT1 homodimer formation at resting condition. Lack of STAT2 enhanced IFNγ-induced *Cxcl10* expression, but has no effect on expression of *Oas1*, an type 1 IFN-induced gene through ISGF3 complex [257]. We speculate that U-STAT1 is responsible for the constitutive expression of *Pnpla2*, *Cpt1a*, *Plin1* and repression of *G0s2* in INS-1 cells. Blocking TYK2 by nifuroxazide may inhibit STAT2 regulatory impact and further stimulate U-STAT1, therefore leading to upregulation of basal *Pnpla2*, *Cpt1a*, *Plin2*, and downregulation of *G0s2* (**Fig. 4-8**). Combined with IFNγ, nifuroxazide further enhanced formation of U-STAT1 and potentiated gene expression of *Pnpla2*, *Cpt1a* and *Plin1*, but attenuated IFNγ-mediated effect on *G0s2* through blocking STAT3. Finally, ruxolitinib strongly upregulated basal *Pgc1a*, suggesting that JAK1/2 play a gatekeeper role in homeostatic regulation of mitochondrial function and lipid metabolism. The specific roles of JAKs/STATs and U-STAT in the constitutive

expression of genes involved in lipid metabolism is intriguing and should be addressed by future research.

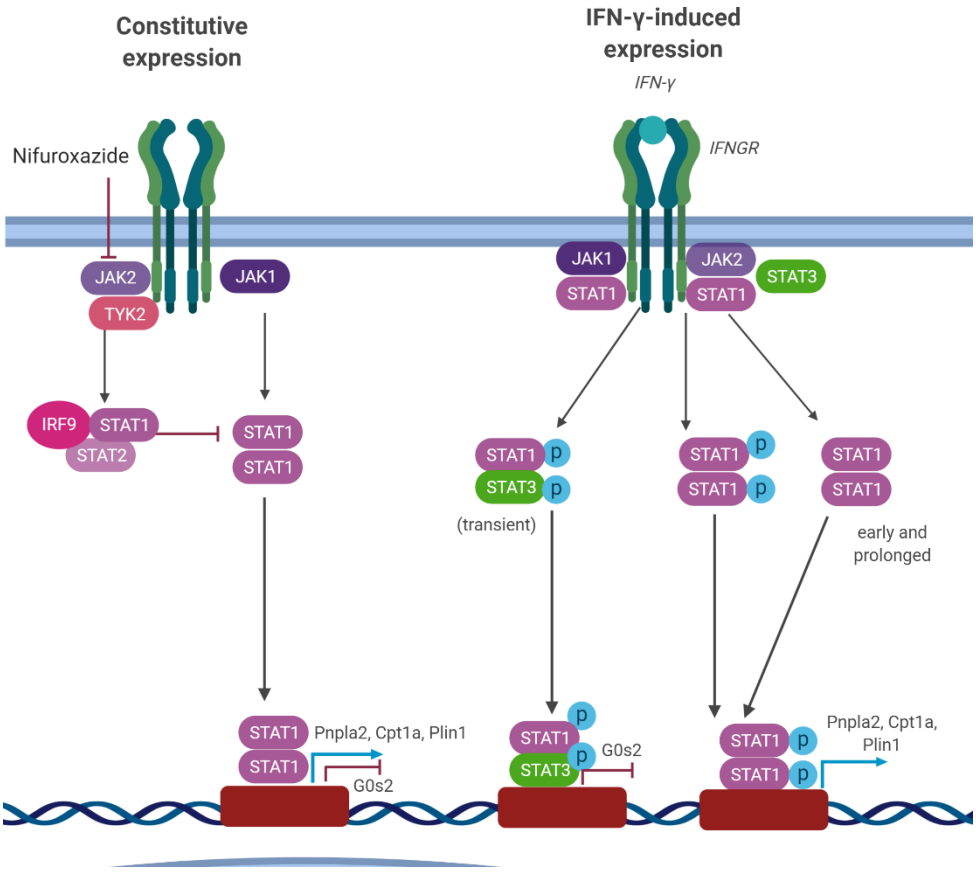


Figure 4-8. Proposed mechanism of JAK/STAT regulation of constitutive and IFNγ-induced expression of lipid metabolism genes.
Basal expression of *Pnpla2*, *Cpt1a*, *Plin1* and *G0s2* is constitutively regulated by unphosphorylated STAT1 (U-STAT1), which is negatively regulated by STAT1-STAT2-IRF9 complex. Blocking JAK2/TYK2 by nifuroxazide inhibits STAT1-STAT2 interaction, therefore enhances transcriptional activation by U-STAT1, further upregulates *Pnpla2*, *Cpt1a*, *Plin1* and downregulates *G0s2*. Upon IFNγ stimulation, STAT3 is transiently phosphorylated and activated, causing downregulation of *G0s2*. IFNγ causes increased levels of phosphorylated STAT1 and U-STAT1, which can be responsible for early and sustained elevation of *Pnpla2*, *Cpt1a* and *Plin1*.

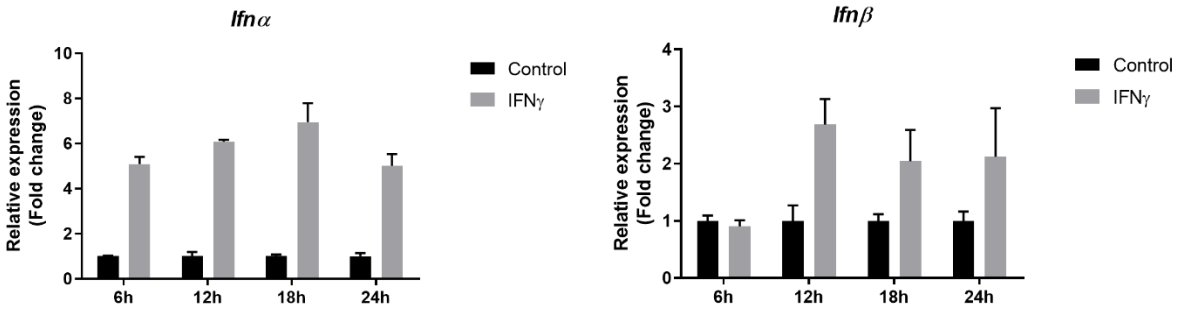
In summary, our data have highlighted the specificity of IFNγ-mediated transcriptional regulation of lipid metabolism in pancreatic β cells. We conclude that IFNγ, compared to IFNα and other proinflammatory cytokines, regulate lipid gene expression in a dynamic manner that is dependent of JAK1/2 signaling. While the transient lipolysis is involved in STAT3-mediated

downregulation of *G0s2*, the early and sustained upregulation of genes involved in lipolysis, LD and FAO by IFNγ can be regulated by crosstalk among multiple JAK/STAT proteins. Future research is therefore necessary to determine the signaling pathways that are responsible for IFNγ and cytokine-mediated metabolic effects in β cells. These studies will be crucial to identify specific targets in order to modulate the effects of cytokines to β cell function and prevent the development of T1D.

APPENDIX

Supplemental Table 4-1. **PCR primer sequences.**

Gene	Protein name	Forward primer (5'-3')	Reverse primer (5'-3')
Cxcl-10	C-X-C Motif Chemokine Ligand 10	AACTGAGGGAGAGCAAACAC	TTTGGGTGATGGAAAGTAGGG
Oas1	2'-5'-oligoadenylate synthetase 1	CAGGAGGTGGAGTTTGATGTG	TCCGTGAAGCAGGTAGAGAA
Stat1	Signal transducer and Activator of	CCAGAGGAGTTTGATGAGATG	CAGGAAGGAATCACAGATGG
Stat3	Signal transducer and Activator of	TCCTGGTATCCCCACTGGTC	CTACCTGGGTCAGCTTCAGG



Supplemental Figure 4-1. IFNγ induces type 1 IFN expression.
INS-1 cells were treated with IFNγ for 6, 12, 18 and 24 h, and mRNA expressions of interferon type 1 *Ifnα* and *Ifnb* were measured. Data are mean ± SD of three replicates per one experiment, shown as fold change to non-treated cells (control) at each timepoint.

Chapter 5. Conclusion, Future Direction and Significance

5.1. Summary of dissertation

Proinflammatory cytokines such as IFNs, TNFα and IL-1β have been shown to alter lipid metabolism in adipocytes, hepatocytes and macrophages-derived foam cells, causing disrupted cellular function and dyslipidemia in metabolic diseases. Current opinions, however, have suggested a physiological role of IFNs in the regulation of FA and cholesterol metabolism in immune cells and certain non-immune cells in response to pathogens, especially viruses. These changes have been linked directly to the activation of immune function and host defense response, indicating complex roles of cytokines in inflammatory diseases. T1D is a chronic autoimmune disease in which insulin-secreting pancreatic β cells are targeted by autoreactive immune cells and elevated cytokines, leading to β cell functional suppression and loss of insulin secretion. Prior to this dissertation, very little was known about the impact of proinflammatory cytokines on β cell lipid metabolism and how they would correlate with alteration of β cell alteration of function. The goal of this dissertation was to investigate the impact of inflammatory cytokines, particularly the pleiotropic cytokine IFNγ, on pancreatic β cell lipid metabolism. IFNγ is secreted by immune cells and possesses dual roles in innate, i.e. anti-viral, and adaptive immunity. While it has been shown that IFNγ potentiates inflammation and β cell dysfunction induced by viral analogs or proinflammatory cytokines, the direct impact of IFNγ on β cell function remains unclear. We hypothesized that IFNγ alters β cell lipid metabolism, which is associated with anti-viral response and functional changes in mitochondria and ER. Below are the highlights of each chapter.

- In chapter 2, we investigated the alteration of islet lipid composition upon islet autoimmunity and discussed the potential role of IFNγ in the regulation of islet TAG levels. The temporal effects of IFNγ on the β cell line INS-1 were examined by non-targeted lipidomics, lipid

droplet staining, and protein/mRNA expression of key genes that regulate FA and cholesterol metabolism.

- Triggering of islet autoimmunity *in vivo* led to sustained IFNγ signaling and elevated levels of TAG in the islets and this preceded insulitis and onset of T1D. These data suggest a novel link between islet autoinflammation and intracellular lipid levels, which can be tied to host defense response.
- IFNγ regulated expression of genes involved in FA and cholesterol metabolism in INS-1 β cell in a dynamic manner. IFNγ transiently downregulated lipolysis inhibitor *G0s2* and upregulated lipase *Pnpla2* and caused a decrease in TAG and LDs levels, suggesting activation of TAG lipolysis.
- In contrast, long term (24 h) exposure to IFNγ increased expression of *de novo* lipogenic genes resulted in elevated levels of NEFA, TAG and LD levels. IFNγ also downregulated genes coding for CE synthesis enzymes and decreased CE levels.
- IFNγ-induced DNL is potentially important to mount a host defense response via enhancing anti-viral gene expression upon PIC treatment. Our results are novel as they describe the metabolic effect of IFNγ on β cells for the first time, and linked to IFNγ-mediated physiological function during immune responses.

➢ In chapter 3, we examined whether the change in intracellular lipids affects β cell mitochondrial and ER function, which can impact cellular stress and insulin secretory function. We concluded that:

- IFNγ upregulated genes regulating mitochondrial carnitine shuttle and β oxidation, but caused a time-dependent downregulation of *Pgc1a* and elevation of acyl carnitines levels. These data suggest IFNγ induces compensation in mitochondrial FAO gene expression in

response to increased lipolysis, but ultimately causes a possible defect in mitochondrial oxidation capacity.

- Indeed, long term exposure to IFNγ is associated with decreased cellular respiration in response to palmitate, indicating impaired mitochondrial FAO.
- IFNγ, however, had minimal impact on glucose-induced OXPHOS, mitochondrial biogenesis and glucose-stimulated insulin secretion.
- The accumulation of TAG by IFNγ was not adequate to cause lipotoxicity, as IFNγ did not cause ER stress by itself. However, changes in lipid metabolism were associated with increased susceptibility to ER stress induced by other proinflammatory cytokines. The data in this chapter suggest that 24 h exposure to IFNγ had small impact on mitochondrial function and unfolded protein response; yet decreases the capacity to oxidize FA and led to the accumulation of acyl carnitines, a marker of mitochondrial oxidation defect. It is likely that chronic exposure to IFNγ *in vivo* will ultimately cause β cell dysfunction, via (1) reducing β cell FAO and FA-mediated amplifying effect on insulin secretion and (2) increasing susceptibility to TNFα and IL-1β-induced toxicity.

➢ Finally, in chapter 4, we determined the signaling pathways that are responsible for IFNγ-mediated effects on gene expression of FA and TAG metabolism. Our results showed that:

- IFNγ regulates gene expression of lipid metabolism in a distinct manner compared to other inflammatory cytokines. In addition, gene expression and LD staining suggest that IL-6 induces lipolysis, while TNFα+IL-1β and IFNα enhance TAG accumulation. These data suggest that proinflammatory cytokines induce TAG accumulation in β cells.
- IFNγ transcriptionally activates lipid metabolism genes through the IFNGR-associated JAK1/2.

- The transient lipolysis is due to phosphorylation and activation of STAT3, however unphosphorylated and phosphorylated STAT1 might be involved in IFNγ-mediated early and sustained upregulation of certain genes involved in lipid metabolism.

5.2. Discussion, limitation and future direction

IFNγ is used frequently in combination with IL-1β and/or TNFα to mimic the inflammatory conditions within the islet microenvironment in T1D. These cytokines induce β cell apoptosis, contributing to loss of β cell mass and insulin secretion [189,258-261]. While these studies attempt to mimic *in vivo* condition, the concentration of each cytokine used in each study is highly variable, leading to difficulty in the interpretation of the results. Particularly, the direct role of IFNγ in β cell dysfunction and the progression to T1D is still unclear. While many studies suggest that IFNγ has "priming" effect, as it potentiates PIC or cytokine-induced gene expression [262], other reports demonstrate a protective role of IFNγ in the progression of autoimmune diabetes. This dissertation provides novel information into how IFNγ regulates β cell FA and cholesterol metabolism and its association with β cell host defense and function.

Our *in vivo* study using type 1 diabetic model LEW.1WR1 rats showed that induction of autoimmune diabetes with administration of PIC led to markedly elevated levels of TAG in pancreatic islets, coinciding with upregulation of IFNγ signaling. In non-obese diabetic mice (NOD), it was shown that islets T cells secret IFNγ, and accumulation of these T cells correlate with acceleration of T1D [263]. In our model, although TAG and IFNγ signaling were elevated before significant infiltration of lymphocytes, it is possible that IFNγ is secreted from infiltrating T cells within acinar cells of the pancreas, or resident T cells in pancreatic lymphnode and acts via paracrine manner. One approach to understand the direct role of IFNγ in LEW.1WR1 islet lipid metabolism would be to use antibody against IFNγ. Alternatively, studies on IFNGR knockout

rodent models, for instance the NOD.IFNγ-/- can also provide significant insight into the role of IFNγ in islet autoinflammation and lipid metabolism.

In vitro studies in INS-1 cells have indicated that IFNγ indeed regulates β cell TAG levels, in part due to the regulation of genes involved in FA metabolism. IFNγ transiently downregulated *G0s2*, upregulated *Pnpla2* and decreased TAG levels, suggesting increased lipolysis. This burst of lipolysis could provide free FAs for membrane synthesis or oxidation for energy demand. Although cellular respiration in response to palmitate at 6 h is not altered by IFNγ, one could argue that the net FAO can be increased due to increased acyl CoAs derived from lipolysis. Particularly, there was an early upregulation of genes regulating carnitine shuttle and β oxidation (*Cpt1a* and *Lcad*) suggesting a compensatory mechanism in response to the rise in acyl CoAs. In order to gain the insight into the fate of lipolysis-derived FAs, cells can be preloaded with fluorescent FAs (BODIPY), then FA trafficking from LD to other cellular organelles could be examined.

In contrast to the early transient lipolysis, IFNγ upregulated lipogenic genes, e.g. *Acca*, *Acly* and *Fasn* at later timepoints, and resulted in elevated NEFA and TAG levels. Major FA species elevated by IFNγ were palmitic acid 16:0 and its derivatives, suggesting enhanced *de novo* lipogenesis. IFNγ treatment also increased TAG levels in the absence of exogenous FAs in the serum, supporting this intepretation. It is noteworthy that despite the upregulation in FAO gene expression, there is a buildup of acyl carnitines, a marker of mitochondrial oxidation defect or overloading the capacity to oxidize FA. It is well known that DNL generates malonyl CoA and allosterically inhibits CPT1a activity. Recent evidence also showed that malonyl CoA can also inhibit CPT2 activity in skeletal muscle [264]. We speculate that CPT1a and CPT2 activities are decreased at 24 h due to increased DNL and may contribute to the accumulation of acyl carnitines. This interpretation is supported by the observation that IFNγ-treated cells had decreased basal and

maximal respiration in response to palmitate, indicative of mitochondrial long chain FAO defect. Collectively, our data suggest IFNγ upregulates DNL in the expense of reduced mitochondrial FAO.

We have demonstrated that upregulation of DNL by IFNγ plays a role in host defense response by potentiating anti-viral gene expression. A central question remaining is the active role of NEFA and LDs in β cell host defense. While the accumulation of LDs cluster at 24 h could be merely reflective of the rise in NEFA flux into storage, it is possible that LDs can actively contribute to IFNγ-mediated anti-viral function. Furthermore, the localization and shapes of LDs induced by IFNγ are intriguing, as they are distinctively different from those induced by TNFα+IL-1β treatment. It is noteworthy that IFNγ upregulates LD surface proteins *Plin1/2/5* while these genes are not affected by other cytokines. The complex roles of PLINs in pancreatic β cell are gaining increasing attention as they have non-redundant function in the regulation of lipolysis, LD biosynthesis and mitochondrial oxidation. It is likely that the regulation of *Plins* by IFNγ contribute to the unique formation and distribution of LDs around the perinuclear region. Understanding the specific role of each PLIN in β cell LD biosynthesis is essential to gain a more profound insight of different effects of proinflammatory cytokines. In addition, studies on LD and ER-mitochondria interaction by transmission electron micrographs will be beneficial to reveal the dynamics of LD synthesis and turnover under proinflammatory cytokines treatment.

IFNγ-induced DNL also raises a question whether DNL generate bioactive lipids for the activation of host defense directly, or FAs themselves are necessary for synthesis of signaling scaffolds. A recent study showed that INS-1 832.13 cells mainly sense dsRNA via MDA5/RIG-1 signaling [265]. While we showed that inhibition of DNL with pharmacological inhibitor of FASN abrogated IFNγ-potentiating effect on gene expression of anti-viral genes, deletion or mutation of

MDA5/RIG-1 signaling and sensing of PIC can be complementary to test whether IFNγ-mediated DNL potentiate the synthesis of mitochondria scaffold. In addition, genetic or pharmacological inhibition of different phases of lipogenesis can be performed to identify the enzymes/metabolites playing the main role in this priming effect.

The main caveat in these studies is the limitation of gene expression data. It is known that many lipid metabolism genes are regulated post-translationally via phosphorylation or acetylation. Regulation of gene expression shown in these studies can result from direct IFNGR activation and caused a proportional change in protein levels (e.g. Pnpla2/ATGL, G0S2), or probably compensatory to elevated FAs (e.g. *Cpt1a*, *Lcad*). Nonetheless, we demonstrated that IFNγ-mediated effects on the expression of lipid metabolism genes is intially dependent on JAK1/2. The involvement of STATs in IFNγ's effects is more complex, and could explain the unique regulation pattern among IFNγ, type 1 IFN, IL-6 and other proinflammatory cytokines. IFNγ was shown to activate STAT3 transiently and activates lipolysis. However, the early and sustained upregulation of *Pnpla2*, *Cpt1a*, *Lcad* and *Plin* seem to involve both phosphorylated and unphosphorylated STAT1 activation. Especially, our data demonstrated a role of JAK/STAT in the constitutive expression of lipid metabolism genes, especially the coactivator of PPARγ (*Pgc1α*), a master regulator of lipid metabolism and mitochondrial biogenesis. The role of JAK/STAT in the constitutive and induced expression of lipid metabolism genes by cytokines and growth factors in pancreatic β cell and other metabolic tissues remain largely unexplored. This potential research area is therefore crucial to identify novel therapeutic targets in metabolic diseases.

5.3. Translational significance

In summary, this dissertation has demonstrated a novel, non-canonical role of IFNγ in lipid metabolism of pancreatic β cells. These effects are shown to be dynamic and orchestrated at least

by transcriptional activation of lipid metabolism genes through the classical JAK1/2 pathway. Importantly, it is the first time that cytokine-induced lipid metabolism is linked to host defense in pancreatic β cells. These studies provide the following translational aspects:

- **The mechanism of actions of proinflammatory cytokines in T1D**

It is well known that proinflammatory cytokines induce apoptosis in pancreatic β cells and loss of insulin secretion. The mechanisms have been shown to involve activation of transcription factor such as STAT1, NF-κB, and upregulation of genes involved in antigen presentation, ER stress, oxidative stress and programmed cell death. However, there has been no report on the effect of proinflammatory cytokines on β cell lipid metabolism. Here we showed that islets under autoinflammation undergo changes in TAG levels, indicating a role of inflammatory mediators in islet lipid metabolism. *In vitro* studies show that long term exposure to IFNγ causes TAG and LD elevation in pancreatic β cells. Similar observation was seen with a mixture of TNFα and IL-1β, however the mechanism at the transcription level as well as the morphology of LDs between two treatments are distinctly different. Importantly, IFNγ-mediated *de novo* lipogenesis is associated with enhanced anti-viral gene expression, suggesting a physiological role of cytokines. However, this comes with an expense: β cells are predisposed to limited mitochondrial oxidation capacity and ER stress. Together these findings have contributed significantly to our knowledge of proinflammatory cytokines' actions in β cell function and their roles in the development of T1D.

- **The effect of cytokine therapies for systemic and pancreatic β cell lipid profiles**

Cytokine therapies, i.e. therapeutics that use cytokines or inhibitors of cytokine action, have been applied for the treatment of autoimmune diseases, infectious diseases, and cancer [266]. Several reports have shown an adverse effect of cytokine therapies to patients' serum/plasma lipid profile. Anti-TNFα therapy was associated with increase in cholesterol and TAG levels in plasma

of patients with rheumatic diseases [267]. IFNα therapy for treatment of HIV and viral hepatitis has been shown to alter plasma triglyceride, cholesterol and lipoprotein levels [61,268]. IFNα therapy can also trigger T1D [269]. Our data demonstrated that proinflammatory cytokines can alter pancreatic β cell TAG and cholesterol ester levels, which are associated with functional change of β cells. Therefore, it is necessary to be aware of cytokine therapies-induced metabolic effect on systemic lipid levels and pancreatic β cell lipid metabolism, which can pose unwanted impact on insulin secretion and sensitivity.

- **The repurpose of lipid-lowering medication for viral-associated diseases**

Targeting lipid metabolism has received enormous attention, especially for repurposing FDA-approved anti-lipid drugs for other diseases. For example, cholesterol-lowering statins have been studied for anti-viral treatment against HCV [270], Zika [271] or Ebola [272]. Anti-obesity drug orlistat was demonstrated to be effective against viral infection [273]. The PPARγ agonist pioglitazone used to treat type 2 diabetes also showed anti-microbial function [274]. As we showed that IFNγ alter FA and cholesterol metabolism in β cells and linked to enhanced anti-viral function, one can evision that modifying this pathway by lipid-targeting drugs could have a beneficial impact on β cell host defense for the prevention of T1D. However, it is noteworthy to keep in mind that inducing DNL can potentially increase the risk of TAG accumulation in β cells and enhance the risk for β cell dysfunction. In addtion, a systemic use of these drugs can cause unpredictable effects on immune system, as shown with PPARγ agonists/antagonists [275]. In conclusion, the results in this study provide the important foundation of targeting lipid metabolism to modify cytokines-mediated effect for T1D. In order to determine the accurate target, however, more systemic studies are required to evaluate the benefits versus the risks of each therapy on β cell function.

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