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MACROPHAGES IN THE PATHOGENESIS OF OBESITY AND INSULIN RESISTANCE-ASSOCIATED ATHEROSCLEROSIS

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Dedication

To my parents and grandparents

for their unconditional love and support in the pursuit of my purpose

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ABSTRACT

Type 2 diabetes (T2D) associates with increased risk for atherosclerosis; however, mechanisms underlying this relationship are poorly understood. Macrophages, which are activated in T2D and causatively linked to atherogenesis, are an attractive mechanistic link. In this study, using a proteomics approach we show that diet-induced obesity and insulin resistance (obesity/IR) modulates a pro-atherogenic, “macrophage-sterol-responsive-network” (MSRN), which in turn, predisposes macrophages to cholesterol accumulation. We identify interferon-gamma (IFNγ) as the mediator of obesity/IR-induced MSRN dysregulation and increased macrophage cholesterol accumulation, and show that obesity/IR primes T cells to increase IFNγ production. Accordingly, myeloid cell-specific deletion of IFNγ receptor (*Ifngr1-/-*) restores MSRN proteins, attenuates macrophage cholesterol accumulation and atherogenesis. Our findings uncouple the strong relationship between hyperinsulinemia and aortic root lesion size in hypercholesterolemic *Ldlr-/-* mice with obesity/IR, but does not affect these parameters in *Ldlr-/-* mice without obesity/IR. Further, we show that obesity/IR-induced IFNγ targets the MSRN through a host defense-independent pathway in macrophages. This pro-atherosclerotic property of IFNγ occurs at ‘metabolic disease-appropriate’ doses without the induction of canonical IFNγ signaling, through a post-transcriptional mechanism. Collectively, our findings identify an IFNγ-macrophage pathway as a mechanistic link between obesity/IR and accelerated atherogenesis.

CHAPTER ONE: INTRODUCTION

Obesity is the leading, global epidemic, defined as a disproportionate body weight for height with an excessive accumulation of adipose tissue that is usually accompanied by mild, chronic, systemic inflammation (González-Muniesa et al., 2017; Williams et al., 2015). According to a 2016 global survey by the World Health Organization (WHO), more than 1.9 billion adults aged 18 years and older are overweight and of these over 650 million adults are obese. In the United States, a survey conducted in 2013-2014 revealed that around 35-40% of the total adult population were obese (Flegal et al., 2016).

The prevalence of obesity is due to a common problem of chronic overnutrition, which over time leads to the development of insulin resistance, wherein insulin becomes less effective at lowering serum glucose levels owing to decreased responsiveness of insulin-target tissues such as liver, adipose and skeletal muscle tissues (Donath and Shoelson, 2011). Obesity and insulin resistance (IR) are further associated with comorbidities such as type 2 diabetes, cardiovascular diseases, some types of cancers and other adverse pathological conditions (González-Muniesa et al., 2017; Williams et al., 2015). The collection of abnormalities associated with the development of type 2 diabetes mellitus and cardiovascular diseases in obese/IR patients is collectively known as metabolic syndrome (Kaur, 2014).

Type 2 diabetes (T2D) is a systemic, metabolic disease caused by progressive failure of pancreatic β-cells to produce sufficient levels of insulin resulting in hyperglycemia (DeFronzo et al., 2015; Donath et al., 2009; Shoelson et al., 2006). The

American Diabetes Association (ADA) declared diabetes to be the 7th leading cause of death in the United States in 2015 with an estimated 30.3 million Americans, or 9.4% of the population diagnosed with this disease.

One of the most common consequences of T2D is the development of macrovascular (e.g. atherosclerosis) and microvascular (e.g. retinopathy, nephropathy, neuropathy, etc.) complications (Beckman et al., 2002; Chait and Bornfeldt, 2009). Atherosclerosis is a progressive disease that develops due to accumulation of lipids in the artery walls causing prolonged inflammation and finally resulting in plaque rupture and blockage of the arteries leading to myocardial infarction or stroke (Lusis, 2000; Weber and Noels, 2011). Although diet and lifestyle, apart from genetic predisposition, primarily influence the rate of development of atherosclerosis, type 2 diabetics have an increased susceptibility for this disease with a 2-4 fold greater risk compared to non-diabetics (Kannel and McGee, 1979). However, the underlying mechanistic processes for this increased risk are poorly understood.

This chapter aims to provide a general framework, highlighting crucial findings in the pathophysiological consequences of metabolic disease, to understand the impact of obesity/IR on macrovascular complications. Further, the work detailed in this dissertation provides mechanistic evidence for the role of obesity/IR in atherosclerosis progression and provides future directions for this research that holds the potential for novel therapeutics to reduce risk of cardiovascular diseases in a highly susceptible type 2 diabetic patient population.

Type 2 diabetes contributes to increased risk for atherosclerosis.

Patients with type 2 diabetes have a 2-4-fold higher risk for developing cardiovascular complications including coronary heart disease (CHD), heart failure and stroke compared to non-diabetics (Gore et al., 2015; Kannel and McGee, 1979). An 18 year long, population-based study comparing CVD risk in type 2 diabetic and non-diabetic patients, deemed T2D as a “coronary heart disease equivalent” (Juutilainen et al., 2005). In this study, the prognosis for type 2 diabetic patients without prior myocardial infarction (MI) was worse than non-diabetic patients with prior MI. The 7-year incidence rate for MI in patients with T2D was 45% whereas it as only 18.8% for non-diabetics (Haffner et al., 1998; Juutilainen et al., 2005). Moreover, the 5-year mortality rate from MI in T2D patients has been shown to be nearly double than that of non-diabetic individuals (Beckman et al., 2002; Herlitz et al., 1998)

Elevated plasma cholesterol levels are a contributor to and a strong predictor of the development of atherosclerosis (Haffner, 1998). Thus, management of this dyslipidemia, particularly high levels of low-density lipoprotein (LDL) with statin therapies have been recommended by the National Cholesterol Education Program (NCEP) and the American Diabetes Association (ADA) over a decade ago, in an effort to reduce risk of CVDs in type 2 diabetic patients (Grundy et al., 1993; Haffner, 1998; Haffner et al., 1998).

Indeed, an epidemiological study based on aggressive lowering of plasma cholesterol levels with simvastatin reported a reduced risk of MI by 55% in diabetics versus a 32% risk reduction in non-diabetic subjects (Pyörälä et al., 1997). Another study reported a 24% reduction in cardiovascular complications in type 2 diabetic

patients that were subjected to simvastatin treatment (Collins et al., 2002). However, in these studies simvastatin treatments were significantly beneficial to non-diabetic subjects as well. Although there have been considerable decreases in cardiovascular events in type 2 diabetic individuals using statin therapies, significant residual CVD risk still remains for these patients (Betteridge, 2011). Thus, though increased plasma cholesterol is one of the major contributors to atherosclerotic progression in T2D patients, other dysfunctionalities in this metabolic syndrome may influence this increased risk these individuals.

There have been many reports regarding the contribution of hyperglycemia to this increased atherosclerotic susceptibility in T2D (Beckman et al., 2002; Chait and Bornfeldt, 2009). However, as demonstrated in the Diabetes Control and Complications Trial (DCCT), glycemic control has shown to limit microvascular complications with little impact on curbing macrovascular diseases in type 2 diabetic patients (1995). Moreover, recent epidemiological studies (ACCORD and ADVANCE) with aggressive glucose-lowering therapies, assessed by reduction in HbA1c, in T2D patients showed either no beneficial effect on CVD risk or resulted in increased mortality rate in these patients (Gerstein et al., 2008; Patel et al., 2008)

Thus, based on epidemiological evidence, hyperglycemia and hyperlipidemia, the two hallmarks of T2D and atherosclerosis respectively, do not solely explain the increased cardiovascular risk observed in diabetic patients.

Mechanisms of increased atherosclerosis risk in T2D patients remain undefined.

Aggressive lowering of risk factors such as high plasma cholesterol and high blood glucose levels do not abrogate the risk for atherosclerosis but resulted in increased mortality in type 2 diabetic patients. Moreover, controlling other risk factors for CVDs such as hypertension and smoking, also do not alleviate this risk (Beckman et al., 2002; Garcia-Touza and Sowers, 2012; Stamler et al., 1993). Thus, traditional risk factors do not explain increased susceptibility of T2D subjects to atherosclerosis.

One of the major challenges in studying T2D-associated atherosclerosis has been to dissociate the effects of obesity-IR and hypercholesterolemia on atherosclerosis progression in these patients. Most diets use to promote atherosclerosis in common animal models such as the *Ldlr-/-* or *Apoe-/-* mice, develop IR along with increased plasma cholesterol levels (Hartvigsen et al., 2007). Hence, an understanding of the direct effects of T2D is crucial as increased cardiovascular complications in these patients cannot be fully explained by hypercholesterolemia alone (Costa et al., 2006).

Macrophages may represent a cellular link between T2D and atherosclerosis.

T2D is an obesity-driven metabolic disease that results in a chronic low-grade systemic inflammation (Shoelson et al., 2006). Infiltration and accumulation of macrophages into the adipose tissue of obese individuals is the main contributor of inflammation in T2D (Ferrante, 2007). Similarly, atherosclerosis also results from inflammatory processes that arise from the prolonged deposition of lipids in the artery wall which leads to lesion formation and ultimately blockage of the arteries (Lusis, 2000; Ross, 1999). Cholesterol-loaded macrophages, called foam cells, are the hallmarks of

atherosclerosis and contribute to the local inflammation within the artery wall (Lusis, 2000; Ross, 1999; Tall and Yvan-Charvet, 2015; Yu et al., 2013). Macrophages are present in almost all major tissues where they have important immunological functions. Since macrophages are key contributors to the pathogenesis of both T2D and atherosclerosis, they could represent an important cellular link between the two diseases. The following sections detail the role of macrophages in (i) promoting insulin resistance in obesity and, (ii) the development and progression of atherosclerosis. These sections aim to provide the rationale for the importance of studying macrophage mechanisms in the context of T2D-associated atherosclerosis progression.

Macrophage-mediated inflammation promotes insulin resistance in obese mice.

The discovery of adipose tissue inflammation as the main mediator of obesity-linked IR by Hotamisligil and colleagues has been pivotal to our current understanding of T2D as an inflammatory disease (Hotamisligil et al., 1993). Briefly, this work was the first to demonstrate the link between inflammation and obesity/IR, where they showed that elevated TNFα levels in adipose tissue as well as in systemic circulation of obese and insulin resistant mice and that neutralization of this pro-inflammatory cytokine resulted in increased insulin sensitivity (Hotamisligil et al., 1993).

Following this, there have been many studies that have identified elevated levels of many pro-inflammatory factors such as IL-6, IL-1β, TGF-β, iNOS, C-reactive protein (CRP) and monocyte chemotactic protein-1 (MCP-1) (Donath and Shoelson, 2011; Gordon and Taylor, 2005) in the adipose tissue and various other studies trying to delineate the inflammatory processes that contribute to obesity-induced IR. One such

seminal work led to the identification of macrophages as the primary immune cells to infiltrate and accumulate in adipose tissue of obese mice (Weisberg et al., 2003). In this study, obese adipose tissue exhibited differential expression of macrophage markers, with 45-60% expression of macrophage F4/80 compared to only 10-15% expression in lean mice (Weisberg et al., 2003). Further, the degree of adiposity was positively correlated with the number of macrophages present in a given depot of adipose tissue of both mice and humans (Weisberg et al., 2003). Another important study demonstrated that the depletion of the pro-inflammatory CD11c+ population of adipose tissue macrophages (ATMs), using a conditional cell ablation system, resulted in a rapid normalization of insulin sensitivity in obese mice (Patsouris et al., 2008).

Phenotypically-varied macrophages populate adipose tissue of lean and obese mice. For instance, pro-inflammatory, classically-activated (M1) macrophages, driven by Th1 mediators such as LPS and IFNγ, are primary found in obese adipose tissue where they exhibit ‘crown-like’ structures around dying adipocytes (Olefsky and Glass, 2010). Alternatively activated (M2) macrophages are found uniformly distributed in lean adipose tissue and exhibit anti-inflammatory functions mediated by Th2 responses that are crucial for the maintenance of insulin sensitivity through IL-10 secretion (Gordon and Taylor, 2005; Odegaard et al., 2007). Moreover, targeting different macrophage activation pathways has also led to alleviation of insulin resistance in obese mice. For instance, myeloid cell depletion of pro-inflammatory signaling molecules such as Iκκ-B, JNK and TLR-4, have been shown to improve insulin sensitivity in obese mice (Arkan et al., 2005; Han et al., 2013; Saberi et al., 2009). Conversely, disruption of repair mechanisms of ATMs such as deletion of macrophage PPARγ, which is necessary to

induce the alternatively-activated, anti-inflammatory M2 phenotype in macrophages, resulted in an increased susceptibility to diet-induced obesity, insulin resistance and glucose intolerance (Odegaard et al., 2007).

More recently, a unique class of pro-inflammatory ATMs called ‘metabolically-activated’ macrophages (MMe) have been discovered in adipose tissue of obese human subjects (Kratz et al., 2014). These MMe macrophages do not exhibit surface markers of classical activation such as those expressed by macrophages isolated from airways of cystic fibrosis patients (Kratz et al., 2014). *In vitro*, ‘metabolic-activated’ phenotype can be observed by treating macrophages with a combination of glucose, insulin and palmitate to recapitulate the *in vivo* setting of metabolic syndrome. In addition to expressing pro-inflammatory markers of classical activation, MMe macrophages also express p62 and PPARγ, proteins that promote lipid metabolism and promote a more M2-like phenotype (Kratz et al., 2014). This duality observed in MMe macrophages represents their cellular plasticity in accordance with the tissue-specific micro-environments in which they are present.

Together, these studies identify macrophages as the key contributors and mediators of inflammation and insulin resistance in obese mice and humans and highlight the importance of their cellular phenotypes and varied mechanisms in response to tissue-specific environments that contribute to the pathogenesis of different diseases.

Macrophages are key mediators in the pathogenesis of atherosclerosis.

Atherosclerosis is a progressive, inflammatory disease of the vasculature that arises from long-term deposition of lipids in the artery wall to form plaques (Moore and Tabas, 2011). The accumulation of lipids in the subendothelial space of the artery wall causes recruitment of blood monocytes which differentiate to macrophages that uptake the deposited lipids within the arterial wall, resulting in the formation of 'foam cells', a hallmark of atherosclerosis. Perpetuated deposition of lipids results in the initiation of inflammatory responses in the sub-arterial space, leading to progression of fatty streaks to advanced plaques in the arteries. These plaques, also known as lesions, in the arteries are typically asymptomatic until the disease reaches advanced stages wherein plaque rupture occurs due to necrosis and subsequent occlusion or blockage of any major arteries results in myocardial infarction (MI) or stroke (Gisterå and Hansson, 2017; Virmani et al., 2002).

Lesion initiation in the artery wall occurs in the form of fatty streaks where lipid is retained in the sub-endothelial space, primarily in the form of apolipoprotein B-containing lipoproteins (ApoB-LPs) interacting with proteoglycans in arterial sites associated with disturbed laminar flow such as arterial bifurcations and branch points (Moore and Tabas, 2011; Shapiro and Fazio, 2017; Williams and Tabas, 1995). Lipoproteins are molecules that consist of a central lipid core, such a cholesteryl fatty acyl esters and triglycerides, surrounded by a layer of phospholipid and proteins (Feingold and Grunfeld, 2000; Moore and Tabas, 2011). Plasma lipoproteins are categorized into seven classes based on their size, lipid composition and type of apolipoproteins. These are chylomicrons, chylomicron remnants, very low-density

lipoproteins (VLDL), intermediate-density lipoprotein (IDL), low-density lipoproteins (LDL), high-density lipoproteins (HDL), and lipoprotein (a) (Lp (a)), where all but HDL are pro-atherogenic (Feingold and Grunfeld, 2000). Apoliproteins play essential roles in lipid metabolism and are also important for the formation and maintenance of the structure of lipoproteins. The main categories of apolipoproteins are ApoA, ApoB, ApoC, and ApoE (Feingold and Grunfeld, 2000). These proteins are synthesized in different organs, with the liver and intestine being the predominant sites of synthesis, and associate with different lipoproteins. For instance, ApoA-I is the main structural component of HDL, whereas ApoB-100 is found in VLDL, IDL and LDL (Feingold and Grunfeld, 2000).

Influx of ApoB-LPs, particularly LDL, in the artery wall causes activation of the endothelial lining resulting in the recruitment of monocytes from the blood stream through secreted chemokines (Moore and Tabas, 2011; Ross, 1999; Shapiro and Fazio, 2017). Extravasation into the subendothelial space initiates the differentiation of blood monocytes in to macrophages, primarily driven by macrophage colony stimulation factor (M-CSF). In the early stages of atherogenesis, macrophages in the artery wall can be found to have droplets of membrane-bound cholesteryl esters, thereby being known as foam cells due to their spongy, 'foam-like' appearance (Yu et al., 2013). After uptake of lipoproteins via scavenger receptors such as scavenger receptor-A (SR-A) on the surface of macrophages (Kunjathoor et al., 2002), cholesteryl esters are hydrolyzed to fatty acids and free cholesterol, which is then transported to the endoplasmic reticulum (ER) (Maxfield and Tabas, 2005). Free cholesterol delivered to the ER has a crucial role in cholesterol metabolism as it leads to downregulation of LDL receptors and a

decrease in endogenous cholesterol synthesis by the sterol-regulatory element binding pathway (SREBP) (Brown and Goldstein, 1997; Goldstein and Brown, 2015). The ER enzyme acyl CoA: cholesterol ester transferase (ACAT) is responsible for re-esterification of free cholesterol to cholesteryl fatty acid esters, that cause the foamy appearance in macrophages (Brown et al., 1980; Goldstein and Brown, 2015).

Reverse cholesterol transport or cholesterol efflux is proposed to be the main mechanism through which macrophages can regulate their cholesterol levels and help reduce cholesterol accumulation in the artery wall. Cholesteryl esters (CE) are hydrolyzed in late endosomes by lipases to release fatty acids and free cholesterol (FC) and these FC droplets are transported within the macrophage to its plasma membrane and efflux occurs via membrane-bound ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1, whose acceptors are apoA-I and HDL, respectively (Yvan-Charvet et al., 2007). The cholesterol-phospholipid efflux to apoA-I forms nascent discoidal HDL that are converted to spherical HDL by the action of lecithin-cholesterol acyltransferase (LCAT) in the plasma, converting FC to CE. This cholesterol-enriched HDL leaves the plaque through the lymphatic system and reaches the liver , where it is taken up by hepatocytes, and either used for the generation of new lipoproteins or is converted to bile and excreted (Röhrl and Stangl, 2013).

Expression of these ABC transporters is controlled by liver X receptor (LXR) transcription factors (LXRα and LXRβ) and deletion or inhibition of these factors or transporters in macrophages result in increased atherogenesis (Calkin and Tontonoz, 2010; Maxfield and Tabas, 2005; Yvan-Charvet et al., 2007). Another family of nuclear receptors, peroxisome proliferator-activated receptors (PPARs), are expressed in

macrophages and other cell types that comprise atherosclerotic lesions and have been extensively studied for their influence on lesion progression (Li et al., 2004). PPARs are involved in regulation of lipid metabolism as well have been shown to exert anti-inflammatory properties in response to NF-κB signaling in monocytes (Jiang et al., 1998; Li and Glass, 2002). Importantly, PPARγ has been shown to regulate cholesterol efflux through regulation LXRα-ABCA1 pathway and absence of PPARγ in macrophages promotes atherosclerotic progression (Chawla et al., 2001).

Continuous exposure to atherogenic lipoproteins causes enhanced inflammatory signaling in macrophages (Moore and Tabas, 2011; Yvan-Charvet et al., 2010). Analysis of artery wall macrophages isolated from animal and human plaques by laser-capture microdissection showed expression of increased inflammatory markers by these cells (Shibata and Glass, 2009). Targeting inflammatory pathways such as NF-κB signaling in macrophages has also resulted in reduced lesion size. Studies wherein deletion of Ικκ-B gene or CD40 receptor, both of which trigger NF-κB activation in macrophages, have been shown to result in decreased macrophage inflammation and lesion areas in the artery wall (Kanters et al., 2003; Lutgens et al., 2010). Thus, macrophage inflammation from prolonged exposure to atherogenic lipoproteins *in vivo* is an important event that potentiates and promotes atherogenesis.

This maladaptive, non-resolving inflammatory response results in a continuous recruitment of blood monocytes into the lesion, which differentiate into macrophages that perpetuate this process in the plaque or atheroma. In addition to monocytes, other immune cells that contribute to the inflammatory response, such as T cells and dendritic cells (DCs), are also recruited to the lesion (Ilhan and Kalkanli, 2015). Failure to

suppress inflammatory cell influx, ineffective clearance of apoptotic cells within the lesion, and decrease in inflammatory cell egress, all contribute to the progression of atherosclerosis (Tabas, 2010). Phagocytic clearance of apoptotic cells is called efferocytosis and is a crucial process in inflammation resolution (Tabas, 2010). This phagocytic process has been shown to occur via tyrosine receptor MerTK signaling and apoptotic ligands and bridging molecules such as Gas6 (Henson et al., 2001). Inefficient efferocytosis results in aggregation of apoptotic cells within the lesion which triggers secondary necrosis, which causes inhibition of anti-inflammatory responses through TGFβ, IL-10 and other such cytokines (Henson et al., 2001). Macrophages secrete matrix metalloproteases (MMPs) and other collagen-degrading factors which, along with necrosis, contributes to 'vulnerable plaques' by causing thinning of the fibrous cap that lines and contains the inflammation within the atheroma (Glass and Witztum, 2001; Tabas, 2010). Prolonged thinning of the fibrous cap leads to plaque instability and rupture. Subsequent blockage of arteries due to thrombus formation, with the help of pro-coagulating and pro-thrombic factors at the rupture site, results in MI, stroke or in severe cases, mortality. Thus, macrophages are involved in all stages of atherosclerosis development and progression and have been extensively studied as a potential target for therapeutic intervention for this non-resolving, inflammatory disease.

The most common animal model used to study atherosclerosis is the *Ldlr-/-* mouse on the WTD. Mice, unlike humans, carry their cholesterol on athero-protective HDL molecules and do not develop atherosclerosis (Getz and Reardon, 2012). Deletion of the LDL receptor (*Ldlr*-/-) results in hypercholesterolemia in these mice due to their inability to clear plasma cholesterol in the absence of *Ldlr*, thereby causing the

development of atherosclerosis. A comparison of peritoneal macrophages from *Ldlr-/-* mice fed either a chow or Western-type diet (WTD) by proteomics analysis revealed a network of highly interconnected proteins, centrally regulated by APOE, called the macrophage sterol-responsive network (MSRN) (Becker et al., 2010). This network was found to be enriched for proteins involved in vesicular-mediated transport, cytoskeletal regulation and lipid binding (Becker et al., 2010). Dysregulation of the MSRN is implicated in atherosclerosis. Treating WTD-fed *Ldlr-/-* mice with interventions such as simvastatin and rosiglitazone, to reduce atherosclerosis, resulted in a correction in the MSRN pattern observed in control WTD-fed *Ldlr-/-* mice and also led to a reduction in macrophage cholesterol accumulation (Becker et al., 2010). Apart from being used as an anti-atherogenic therapy, rosiglitazone, a thiazolidinedione (TZD), is commonly used in type 2 diabetic patients for improving insulin-sensitivity (Kudzma, 2002). The ability of rosiglitazone to correct the MSRN in WTD-fed *Ldlr-/-* macrophages implies the potential involvement of this macrophage protein network in obesity/IR-associated atherosclerosis. Therefore, evaluation of the effects of obesity/IR on artery wall macrophages and its impact on the MSRN could provide an understanding of the mechanisms through which obesity/IR enhance the increased risk for atherosclerotic progression.

CHAPTER TWO: OBESITY AND INSULIN RESISTANCE PROMOTES ATHEROSCLEROSIS THROUGH AN IFNγ-REGULATED MACROPHAGE PROTEIN NETWORK

Introduction

Compared to non-diabetics, patients with T2D have an 2-4 fold increased risk for cardiovascular diseases (CVD), with a greater overall plaque burden and a higher rate of multi-vessel disease, during their lifetime (Beckman et al., 2002; Gore et al., 2015; Haffner et al., 1998; Hayward et al., 2015). Additionally, the 7-year incidence of first myocardial infarction (MI) or death in type 2 diabetic patients is ~6-fold higher than in non-diabetics and the 5-year mortality rate following an MI is nearly double for patients with T2D compared to non-diabetics (Haffner et al., 1998).

Despite a ~70% mortality rate due to cardiovascular diseases such as atherosclerosis (Laakso, 2010), the underlying mechanisms for this increased risk for T2D-associated CVD is poorly understood. Based on epidemiological studies, traditional risk factors for developing CVD such as smoking, hypertension and LDL cholesterol, total cholesterol and triglyceride levels, do not explain the increased risk associated with developing CVD in type 2 diabetic patients (Beckman et al., 2002; Gore et al., 2015; Haffner et al., 1998; Hayward et al., 2015). Moreover, additional studies with intervention therapies showed increased mortality is observed with aggressive glycemic control, lowering of plasma cholesterol levels with stain treatment and controlled hypertension (Banach et al., 2016; Gore et al., 2015).

Macrophages may represent an important cellular link between T2D and atherosclerosis. Macrophages are inappropriately activated during obesity and insulin resistance (IR) and contribute largely, along with other immune cells to obesity/IR-induced inflammation (Chawla et al., 2011; Kratz et al., 2014; McNelis and Olefsky, 2014). Macrophages are also involved in all stages of atherosclerosis development and are causatively linked to the initiation, progression and rupture of atherosclerotic plaques (Li and Glass, 2002; Moore et al., 2013; Tabas and Bornfeldt, 2016). For example, the inability of macrophages to clear cholesterol leads to the formation of foam cells, their defective clearance of apoptotic cells in the artery wall promotes necrotic core formation and increases plaque complexity, and their increased secretion of proteases destabilizes atherosclerotic plaques and promotes plaque vulnerability (Li and Glass, 2002; Moore et al., 2013; Tabas and Lichtman, 2017). Although many of the macrophage proteins involved in each of these critical functions are known, and changes in their levels in the plaques of atherosclerotic mice (and in some cases humans) have been documented (Ait-Oufella et al., 2007; Fazio et al., 2002; Hu et al., 2006; Overton et al., 2007), the mechanisms driving their dysregulation *in vivo* are incompletely understood. Thus, taken together, the idea that obesity/IR alters macrophages in a way to promote atherosclerosis is an attractive hypothesis.

However, the challenge with testing this hypothesis experimentally is that standard high-fat and high-cholesterol diets that produce hypercholesterolemia in atherosclerotic mice also result in a concomitant development of obesity/IR and conversely, diets that promote obesity/IR also result in elevated plasma cholesterol levels in these mice. Thus, it has been difficult to dissociate the direct and indirect

effects of obesity/IR on atherosclerotic progression in the context of hypercholesterolemia, such as in the *Ldlr-/-* or *Apoe-/-* mice fed the WTD.

In order to overcome this challenge, we used a combination of genetic and dietary strategies to study macrophages in the context of either hypercholesterolemia or obesity/IR alone, or in combination. Using an unbiased proteomics approach, we have identified that obesity/IR via IFNγ, targets a pro-atherogenic macrophage protein network called the macrophage sterol-responsive network or MSRN (Becker et al., 2010) to promote foam cell formation. Additionally, we show that blocking macrophage IFNγ signaling (by deleting *Ifngr1* in myeloid cells) in *Ldlr-/-* mice resulted in a normalization of the MSRN and attenuated foam cell formation and atherosclerosis, only in the presence of obesity/IR. Collectively, our studies demonstrate that obesity/IR-induced IFNγ promotes macrophage dysfunction to promote foam cell formation and atherosclerosis.

Materials and Methods

Mice: All animal studies were approved by the University of Chicago IACUC (ACUP#72209). Wild-type (CD45.1 or CD45.2), *Ldlr-/-*, and *Ifngr1-/-* mice on the C57BL/6 background were purchased from Jackson Labs. For DIO studies, wild-type (*wt*) and *Ifgnr1-/-* mice were placed on a low-fat (20:50503, PicoLab) or 60% high-fat diet (D12492, Research Diets Inc.) for 9 weeks. For atherosclerosis studies, *Ldlr-/-* mice or chimeric mice were placed on a low-fat (20:50503, PicoLab), low-fat high cholesterol (TD02026, Envigo), or western-type diet (TD96121, Harlan Teklad) diet for up to 16 weeks.

Blood measurements: Serum insulin levels were measured by ELISA (Millipore) and blood glucose levels were measured with a One Touch Ultra 2 glucometer (Lifescan) following a 3h fast. Total plasma cholesterol levels and cholesterol levels within the VLDL, LDL, and HDL fractions were obtained by the Amplex Red Cholesterol Assay kit (Invitrogen). Lipoproteins were separated by fast protein liquid chromatography on two Superose 6 size exclusion columns in tandem (GE Lifescience).

Bone marrow transplantation: Bone marrow cells ($5x10^6$) collected by PBS perfusion of the femurs and tibia of *Ifngr1-/-* or *wt* male mice were injected into the retro-orbital sinus of lethally irradiated (9.5 Gy ionizing radiation) male *Ldlr-/-* recipient mice. For *wt* transplants, engraftment was determined by flow cytometry using the ratio of CD45.1 (donor) to CD45.2 (recipient) positive bone marrow cells. For *Ifngr1-/-* transplants, since both donor and recipient were on the CD45.2 background, engraftment was quantified by PCR as the ratio of *Ifngr1-/-* (donor) to *wt* (recipient) signal. Mice were on antibiotics for 1 week before transplants, 2 weeks after transplant, and allowed to recover for 6 weeks before initiation of diets.

Quantification of atherosclerosis: Anesthetized mice were perfused with PBS followed by 4% paraformaldehyde with 5% sucrose in PBS. The heart and upper vasculature were excised, cleaned of adventitia, and imbedded in optimal cutting temperature (OCT), and serial 10-μm sections in the aortic root were collected as previously described in (Sontag et al., 2014). Sections, beginning at the appearance of

the coronary artery and aortic valve leaflets, were stained with Oil Red O/Fast green, and digital images were captured using a Nikon Eclipse Ti2 microscope. Aortic root lesion area was quantified by cross-sectional analysis of four sections/mouse (spaced 100 μm apart) using NIS Elements AR software.

Isolation of macrophages: Bone marrow-derived macrophage isolation and activation – Bone marrow cells were isolated from the femur and tibia of wildtype C57/Bl6 wildtype mice (Jackson Labs) and pooled. Cells were plated in Roswell Park Memorial Institute medium (RPMI) containing 10% FBS and 30% L-conditioned media. Media was changed on day 3 and 5, with activation occurring on day 6. For activation, cells were treated with IFNγ (12ng/mL; R&D Biosystems) in 30% L-conditioned media made with 10% FBS containing-RPMI, for 24h. For activation, cells were treated with IFNγ (12ng/mL; R&D Biosystems) in 30% L-conditioned media made with 10% FBS containing-RPMI for 24h. Macrophage-conditioned media were prepared by treating cells with glucose and/or insulin or cytokines for 24 hr, washing cells with PBS, and incubating them in serum-free media for an additional 24 hr. Protein lysate was collected in 1% SDS and quantified using a Pierce BCA protein assay kit (ThermoScientific). MSRN proteins in the macrophage-conditioned media was measured and normalized to total cellular protein, which were both quantified by immunoblotting.

Peritoneal macrophage isolation and activation – Peritoneal macrophages were harvested from the mice 5 days after 4% thioglycolate was injected. Cells were washed with phosphate-buffered saline (PBS), seeded into 24-well plates (0.5×10^6/well),

incubated at 37°C for 2 h in serum-free Dulbecco's minimum essential medium (DMEM), and washed 3 times with PBS. Macrophages were then cultured for 24h in DMEM, following which they were subjected to different treatments. Cells from diet-fed mice were either treated or their protein lysate was harvested 2h post-plating following isolation.

Aortic macrophages isolation – Aortic macrophages were isolated from *Ldlr-/-* fed a western diet for 16 weeks. Animals were injected IP with PBS or IFNγ (25 µg/Kg = 100U/kg) (Whitman et al., 2000), 48h and 24h prior to euthanasia. Anesthetized mice were perfused with cold PBS with 20 U/ml heparin and the upper vascular isolated and dissected of adipose tissue and para-aortic lymph nodes. The intima in the arch and innominate artery were excised and incubated in digestion media in HBSS for 1 hour (Butcher et al., 2011). Macrophages were isolated using anti-CD11b microbeads (Miltenyl Biotek) and plated in 96-well dish in serum-free DMEM for 24 hours.

Macrophage cholesterol loading: Macrophages were incubated for 24 hr in serum-free medium supplemented with or without 2% serum from *Ldlr*−/− mice fed a WTD for 12 weeks. Cellular lipids were extracted using isopropanol, dried, and solubilized in methanol:chloroform (2:1), and proteins were solubilized with 0.1% NaOH. Cholesterol levels were quantified using an Amplex Red Cholesterol Assay Kit (Invitrogen) and normalized to total cellular protein, which was measured using a Pierce BCA protein assay kit (ThermoScientific).

Protein isolation for proteomics: Macrophage-conditioned medium (~10 µg protein/mL) was collected, clarified by centrifugation (5 min at 1,000×*g*), supplemented with 0.02% sodium deoxycholate and 20% trichloroacetic acid, and incubated overnight at 4°C. Proteins were harvested by centrifugation (15,000×*g* for 30 min at 4°C). The protein pellet was washed twice with ice-cold acetone, reconstituted in digestion buffer (0.1% Rapigest (Waters Corp.), 50 mM Tris-HCl, pH 8.8), and then reduced, alkylated, and digested overnight at 37°C with sequencing-grade trypsin (1:50, w/w, trypsin/protein; Promega). Tryptic digests were mixed with acetic acid (1:1, v/v) and subjected to solid-phase extraction on a C18 column (HLB, 1 mL; Waters Corp.) according to the manufacturer's protocol. Fractions containing peptides were dried under vacuum and resuspended in 0.3% acetic acid/5% acetonitrile (1 mg protein/mL) for analysis. Proteins considered for analysis had to be identified in at least 4 of 5 replicates for one biological condition. Differentially expressed proteins were identified using a combination of the t-test ($p<0.05$) and G-test ($G>1.5$) as previously described (Heinecke et al., 2010).

Liquid chromatography-electrospray ionization-tandem MS (LC-ESI-MS/MS): Tryptic digests (2 µg protein) were injected in duplicate into a trap column (Paradigm Platinum Peptide Nanotrap, 0.15×50 mm; Michrom Bioresources, Inc.) and desalted for 5 min with 5% acetonitrile, 0.1% formic acid (50 µL/min). Peptides were then eluted onto an analytical reverse-phase column (0.150×150 mm, 5 µm beads; Magic C18AQ, Michrom Bioresources, Inc.) and separated at a flow rate of 1 µL/min over 180 min, using a linear gradient of 5% to 35% buffer B (90% acetonitrile, 0.1% formic acid) in

buffer A (5% acetonitrile, 0.1% formic acid). Mass spectra were acquired in the positive ion mode, using electrospray ionization and a linear ion trap mass spectrometer (LTQ, Thermo Electron Corp.) with data-dependent acquisition (Vaisar et al., 2007). MS/MS scans were obtained on the 8 most abundant peaks in each survey MS scan.

Peptide and protein identification (Shotgun proteomics): MS/MS spectra were searched against the mouse International Protein Index (IPI) database (version 2006/04/18) (Kersey et al., 2004), using the SEQUEST search engine with the following search parameters: unrestricted enzyme specificity, 2.8 amu precursor ion mass tolerance, 1.0 amu fragment ion mass tolerance, fixed Cys alkylation, and variable Met oxidation. SEQUEST results were further validated with PeptideProphet (Keller et al., 2002) and ProteinProphet (Nesvizhskii et al., 2003), using an adjusted probability of ≥0.90 for peptides and ≥0.96 for proteins. Proteins considered for analysis had to be identified in every biological replicate of at least one biological condition. When MS/MS spectra could not differentiate between protein isoforms, all isoforms were included in the analysis.

Targeted proteomics: Proteins of interest were quantified using liquid chromatography Parallel Reaction Monitoring (PRM) MS on a Thermo Orbitrap Fusion Lumos tribrid mass spectrometer (Themo Scientific) connected to a NanoACQUITY HPLC (Waters). Several peptides from each proteins of interest were monitored by selecting their precursor ions in the quadrupole analyzer (selection window 1.6 Da) and full scan MS/MS after HCD fragmentation (NCE 29%) in the Orbitrap analyzer with high

resolution (15,000). Data were processed using Skyline software (MacLean et al., 2010). Identity of the chromatographic peaks was ascertained by matching the PRM MS/MS spectrum to the spectra from the shotgun experiment (dot product > 0.9 and mass precision < 5 ppm).

Protein quantification and statistical analysis: Proteins detected by LC-ESI-MS/MS were quantified by spectral counting (the total number of MS/MS spectra detected for a protein). Two replicate injections for each sample were averaged to obtain spectral counts. Differences in relative protein abundance were assessed with the *t*-test and *G*-test. Permutation analysis was used to empirically estimate the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Significance cutoff values for the *G*-statistic and *t*-test were determined using PepC.

IFNγ production by T cells: Isolated splenocytes ($0.05x10^6$ cells) were treated with anti-CD3 and anti-CD28 antibodies (eBioscience/Invitrogen) to activate T cells. After 48 hours, IFNγ levels in the media were measured using a Mouse IFNγ Ready-Set-Go ELISA kit (eBioscience/Invitrogen).

Immunoblot analysis: Macrophage-conditioned medium was subjected to SDS-PAGE on 4%–12% gradient gels, transferred to PVDF membranes, and probed antibodies (0.5 µg/mL). Proteins were visualized using the LiCor Odyssey Imaging system and quantified by densitometry, using ImageJ software.

Flow cytometry: Fluorochrome labeled cells were analyzed according to the workflows presented in (Figures 2.2 and 2.14). Analyses were conducted using a Canto-II or LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo software v.9.4.11. Protein levels were quantified by mean fluorescence intensity and normalized to isotype to facilitate comparison between samples.

Antibodies for flow and immunoblotting: For flow cytometry: anti-CD45.1, anti-CD45.2, anti- CD11b, anti-F4/80, and anti-CD11c (eBioscience/Invitrogen). For immunoblotting: rabbit anti-APOE (Abcam), goat anti-MFGE8 (R&D Systems), and rabbit anti-STAT1, rabbit anti-phospho-STAT1 (701) and rabbit anti-GAPDH (Cell Signaling).

qRT-PCR: RNA was isolated using QIAGEN Midi-Prep Kits and RT with Quantiscript (QIAGEN) using random hexamers (Invitrogen). mRNA levels were measured with specific primers (**Table 2.1**) using SYBR green on a One Step Plus system (Applied Biosystems). Relative levels of each target gene were calculated using the ΔΔCt formula and 18S RNA as a control.

Statistics: For proteomics studies, differentially expressed proteins were identified using a combination of the unpaired two-tailed Student's *t*-test ($p < 0.05$) and *G*-test ($G > 1.5$) with correction for false discovery (FDR < 5%) as previously described (Heinecke et al., 2010). For all other studies, statistical significance ($p < 0.05$) was

determined by an unpaired two-tailed Student's *t*-test using Prism GraphPad software (v.6.0h). Data are presented as means ± SEM.

Gene	Primer	Primer Sequence
Ifrngr1	Wild type	TCGCTTTCCAGCTGA
	Deficient	CTCGTGCTTTACGGTATCGC
	Common	CCACCTCAGCACTGTCTTCA
Srebp2	Forward	GTTGACCACGCTGAAGACAGA
	Reverse	CACCAGGGTTGGCACTTGAA
Abca1	Forward	GCTTGTTGGCCTCAGTTAAGG
	Reverse	GTAGCTCAGGCGTACAGAGAT
Sra1	Forward	TTCACTGGATGCAATCTCCAAG
	Reverse	CTGGACTTCTGCTGATACTTTG
Cd36	Forward	ATGGGCTGTGATCGGAACTG
	Reverse	GTCTTCCCAATAAGCATGTCTCC
Abcg1	Forward	GTGGATGAGGTTGAGACAGACC
	Reverse	CCTCGGGTACAGAGTAGGAAAG
Lxra	Forward	ACAGAGCTTCGTCCACAAAAG
	Reverse	GCGTGCTCCCTTGATGACA
Acta1	Forward	CCCAGACATCAGGGAGTAATGG
	Reverse	TCTATCGGATACTTCAGCGTCA
Cdh5	Forward	CCACTGCTTTGGGAGCCTT
	Reverse	GGCAGGTAGCATGTTGGGG
Cd11b	Forward	CCATGACCTTCCAAGAGAATGC
	Reverse	ACCGGCTTGTGCTGTAGTC
Irf1	Forward	GCTGGAGTTATGTCCCTTTCCATATC
	Reverse	GGACTCAGCAGCTCTACCCTACCT
Irf8	Forward	GCTGGTTCAGCTTTGTCTCC
	Reverse	GATCGAACAGATCGACAGCA
Ibp1	Forward	GTGTGGTAGAAGCCCACTATTGC
	Reverse	CCACATGAAAGGCCCAGTGTGC

Table. 2.1: List of primer sequences for PCR

Results

Obesity/IR targets a pro-atherogenic macrophage protein network and promotes foam cell formation.

In order to determine the direct effects of obesity/IR on atherogenesis, it is important to study the impact of obesity/IR on macrophages in a normocholesterolemic environment and compare it to those from hypercholesterolemic, atherosclerotic mice.

To do so, we used a combination of genetic models and dietary interventions that induced conditions of (i) '*obesity/IR only*', (ii) '*hypercholesterolemia (HC) and obesity/IR*' and (iii) '*HC only*' (Hartvigsen et al., 2007) **(Figure 2.1)**. In addition, we used a proteomics approach to determine how these various conditions affected the protein abundance patterns in elicited peritoneal macrophages, which have been demonstrated to be reasonably surrogates for artery wall macrophages *in vivo* (Becker et al., 2010; Li et al., 2004).

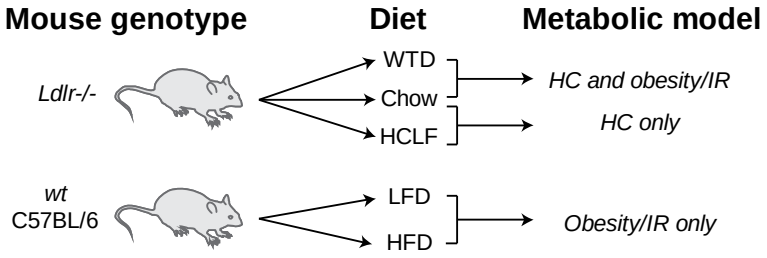


Figure. 2.1: Genetic and Dietary models to study impact of obesity/IR on atherosclerosis. '*Obesity/IR only*' model: *wt* C57BL6 mice were fed a low-fat (LFD) or high-fat diet (HFD) for 9 weeks. '*HC ± obesity/IR*' models: *Ldlr-/-* mice were fed a chow, western-type diet (WTD), or high-cholesterol low-fat (HCLF) diet for 12 weeks.

To determine whether obesity/IR is sufficient to alter macrophages to promote atherosclerosis, we started with the '*obesity/IR only*' model where C57BL/6 mice were fed a low-fat (LFD) or high-fat (HFD) for 9 weeks, following which peritoneal macrophages were isolated from these mice. Purity of isolated peritoneal macrophages was determined by flow cytometry using CD11b and F4/80 antibodies (**Figure 2.2**). Metabolic parameters such as body weight, fasting glucose, insulin and plasma cholesterol are significantly increased in HFD-fed mice compared to the control group (**Figure 2.3A**). Proteomic analysis of conditioned media from purified peritoneal

macrophages identified 27 secreted proteins that were altered by obesity/IR (FDR<5%) (**Figure 2.3B-C).** Nine of those proteins resided in a pro-atherogenic, “macrophage sterol-responsive network” (MSRN) (**Figure 2.3B, Table 2.2**) we previously identified in foam cells from hypercholesterolemic *Ldlr-/-* mice (Becker et al., 2010).

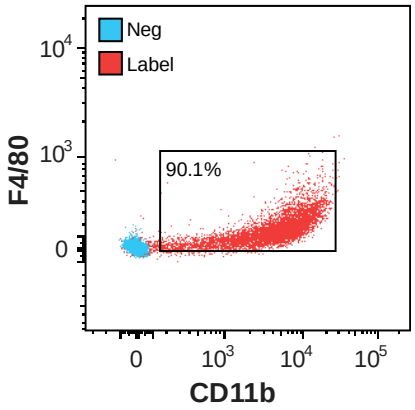


Figure. 2.2: Analysis of peritoneal macrophage purity. Control or genetically modified mice fed the various diets were injected with thioglycolate in the peritoneal cavity, and elicited peritoneal macrophages were isolated five days after injection. Peritoneal macrophage purity was confirmed by flow cytometry using antibodies raised against murine F4/80 and CD11b. A representative image is shown.

Although the MSRN was previously identified as “sterol-responsive” as dysregulation of the network was observed when macrophages were sterol loaded *in vivo* (Becker et al., 2010), the protein changes observed in the ‘*obesity/IR only*’ model occurred independent of any changes in macrophage cholesterol and triglyceride levels (**Figure 2.3D**), suggesting that a significant portion of this network could be regulated by obesity/IR.

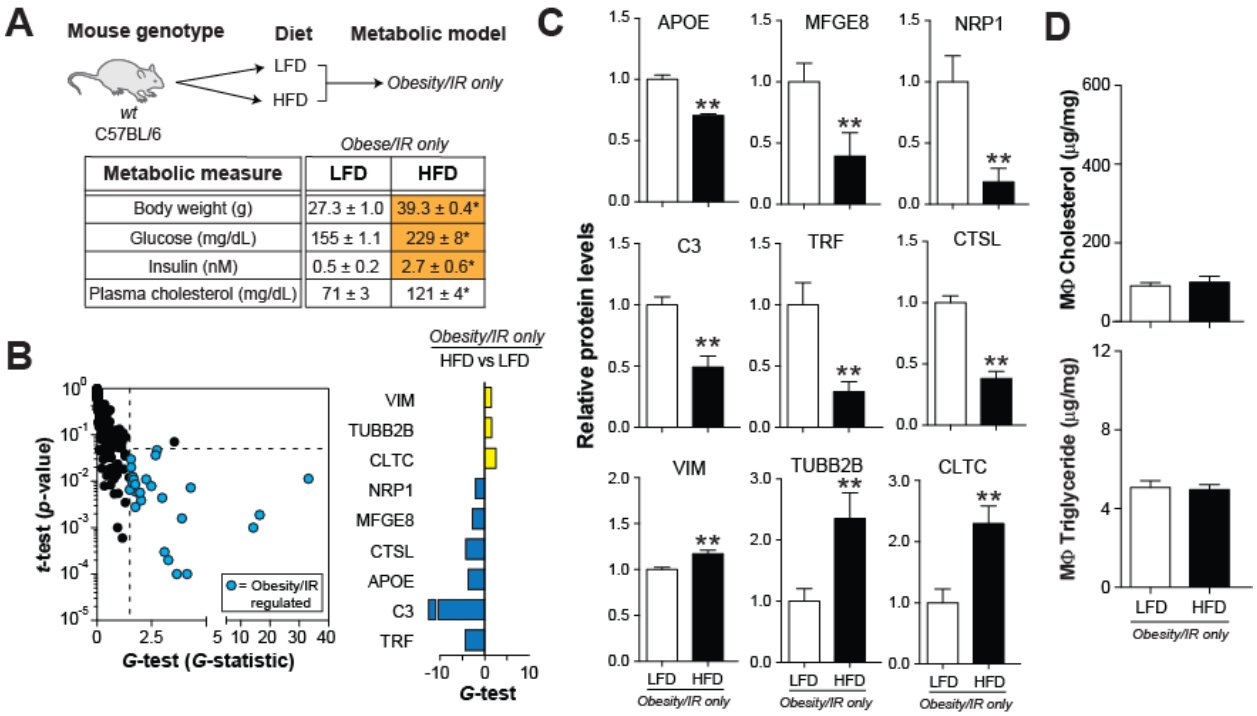


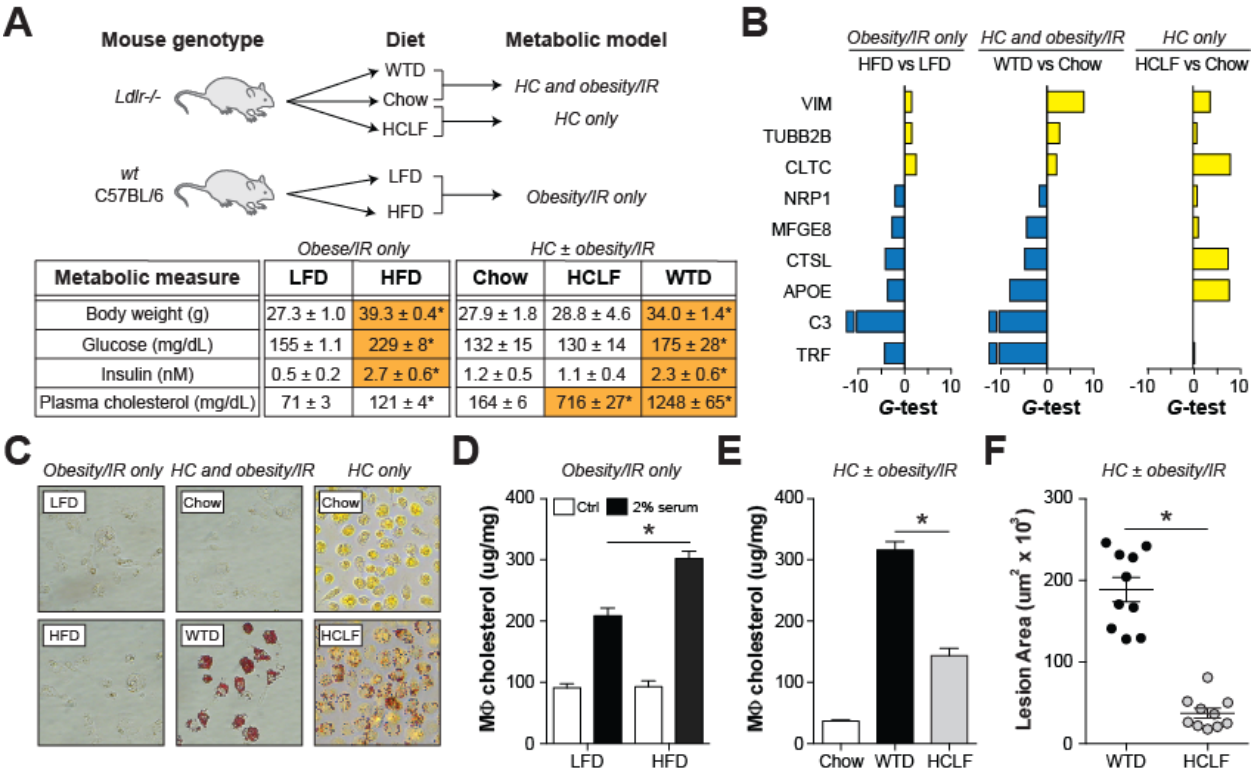
Figure. 2.3: Obesity/IR targets the MSRN, a pro-atherogenic macrophage protein network. '*Obesity/IR only*' model: *wt* C57BL6 mice were fed a low-fat (LFD) or high-fat diet (HFD) for 9 weeks. *Panel A*: Metabolic parameters. *Panel B:* Proteomics analysis of peritoneal macrophages. Differentially expressed proteins (blue circles) were identified based on the *G*-test (*G*>1.5) and *t*-test ($p<0.05$). *Panel C*: Regulation of 9 MSRN proteins. *Panel D*: Peritoneal macrophage cholesterol and triglyceride levels. Results are mean ± SEM. *, $p<0.05$ (*t*-test). **, *G*>1.5 (*G*-test) and $p<0.05$ (*t*-test). n=2-5/group.

Obesity/IR only Model						
Protein annotations			*wt* C57BL/6 mice HFD vs. LFD		*Ifngr1-/-* C57BL/6 mice HFD vs. LFD	
Uniprot	Entrez	Protein	G-test (HFD : LFD)	t-test	G-test (HFD : LFD)	t-test
P01027	12266	C3	-16.43	0.0019	0.57	0.4722
Q921I1	22041	TRF	-4.30	0.0073	0.00	0.9792
P06797	13039	CTSL	-4.13	0.0001	3.80	0.0070
P08226	11816	APOE	-3.66	0.0001	0.00	0.9646
P21956	17304	MFGE8	-2.69	0.0368	0.49	0.1593
P97333	18186	NRP1	-2.02	0.0039	0.49	0.0509
P20152	22352	VIM	1.50	0.0066	-0.50	0.0820
B2RSN3	73710	TUBB2B	1.56	0.0201	ND	ND
Q68FD5	67300	CLTC	2.49	0.0079	1.04	0.0035

Table 2.2: Shotgun proteomic analysis of elicited peritoneal macrophages from '*Obesity/IR only*' model. Proteomics analysis of the conditioned media collected from elicited peritoneal macrophages isolated from *wt* or *Ifngr1-/-* C57BL6 mice fed the LFD or HFD diets. Proteomics data were analyzed by the G-test (G-statistic) and t-test (p-value). Differentially abundant proteins are shaded; red = up-regulated in 1st sample relative to the 2nd, green = down-regulated in the 1st sample relative to the 2nd. ND = not detected. n=3-5 mice/group.

Next, we asked whether obesity/IR was necessary to produce changes in the MSRN in the hypercholesterolemic *Ldlr-/-* mice. Analysis of the metabolic parameters in the different models showed a significant increase in body weight, fasting glucose and insulin in the '*obesity/IR only*' and '*(HC) and obesity/IR*' models but not '*HC only*' model (**Figure 2.4A)**. Compared to the '*obesity/IR only*' model, all of the 9 MSRN proteins were similarly dysregulated in the '*HC and obesity/IR model*', but most were normalized or counter-regulated in the '*HC only*' model (**Figure 2.4B, Table 2.2, Table 2.3**) even though the macrophages were cholesterol loaded (**Figure 2.4C**). For example, APOE levels were lowered in western-type diet (WTD)-fed *Ldlr-/-* mice but raised in high-cholesterol-low-fat (HCLF) diet-fed mice, The results observed in the 'HC only' model are in agreement to the *in vitro* findings that APOE levels are increased when

macrophages are loaded with cholesterol (**Figure 2.5**) (Basu et al., 1981), suggesting that *in vitro* cholesterol loading does not mimic MSRN protein changes observed *in vivo*.



Metabolic measure	Obese/IR only: LFD	Obese/IR only: HFD	HC ± obesity/IR: Chow	HC ± obesity/IR: HCLF	HC ± obesity/IR: WTD
Body weight (g)	27.3 ± 1.0	39.3 ± 0.4*	27.9 ± 1.8	28.8 ± 4.6	34.0 ± 1.4*
Glucose (mg/dL)	155 ± 1.1	229 ± 8*	132 ± 15	130 ± 14	175 ± 28*
Insulin (nM)	0.5 ± 0.2	2.7 ± 0.6*	1.2 ± 0.5	1.1 ± 0.4	2.3 ± 0.6*
Plasma cholesterol (mg/dL)	71 ± 3	121 ± 4*	164 ± 6	716 ± 27*	1248 ± 65*

Figure. 2.4: Obesity/IR is necessary to produce MSRN dysregulation and promote macrophage cholesterol loading. '*Obesity/IR only*' model: *wt* C57BL6 mice were fed a low-fat (LFD) or high-fat diet (HFD) for 9 weeks. '*HC ± obesity/IR*' models: *Ldlr-/-* mice were fed a chow, western-type diet (WTD), or high-cholesterol low-fat (HCLF) diet for 12 weeks. *Panel A*: Metabolic parameters. *Panel B:* Proteomics analysis of peritoneal macrophages. Differentially abundant proteins (yellow = up, blue = down) were identified based on the *G*-test (*G*>1.5) and *t*-test ($p<0.05$). *Panel C*: Oil-red-O staining of peritoneal macrophages. *Panel D*: Cholesterol levels in peritoneal macrophages treated with/without 2% serum from WTD-fed *Ldlr-/-* mice. *Panel E*: Cholesterol levels in freshly isolated peritoneal macrophages. *Panel F*: Aortic root lesion area. Results are mean ± SEM. *, $p<0.05$ (*t*-test). n=5-10/group.

HC +/- Obesity/IR Model						
Protein annotations			*Ldlr-/-* mice WTD vs. Chow		*Ldlr-/-* mice HCLF vs. Chow	
Uniprot	Entrez	Protein	G-test (WTD : Chow)	t-test	G-test (HCLF : Chow)	t-test
P01027	12266	C3	-9.80	0.0036	-0.01	0.8893
Q921I1	22041	TRF	-2.90	0.0082	0.31	0.2999
P06797	13039	CTSL	-4.46	0.0458	7.54	0.2722
P08226	11816	APOE	-12.72	0.0047	7.75	0.1559
P21956	17304	MFGE8	-3.03	0.0271	1.06	0.5212
P97333	18186	NRP1	-1.84	0.0013	0.85	0.4994
P20152	22352	VIM	3.57	0.0052	3.66	0.2052
B2RSN3	73710	TUBB2B	4.06	0.0169	0.78	0.4146
Q68FD5	67300	CLTC	2.63	0.0500	8.01	0.1054

Table 2.3: Shotgun proteomic analysis of elicited peritoneal macrophages from '*HC ± obesity/IR*' model. Proteomics analysis of the conditioned media collected from elicited peritoneal macrophages isolated from *wt* or *Ifngr1-/-* C57BL6 mice fed the LFD or HFD, from *Ldlr-/-* mice fed the chow, WTD, or HCLF diet. Proteomics data were analyzed by the Gtest (G-statistic) and t-test (p-value). Differentially abundant proteins are shaded; red = up-regulated in 1st sample relative to the 2nd, green = down-regulated in the 1st sample relative to the 2nd. ND = not detected. n=3-5 mice/group.

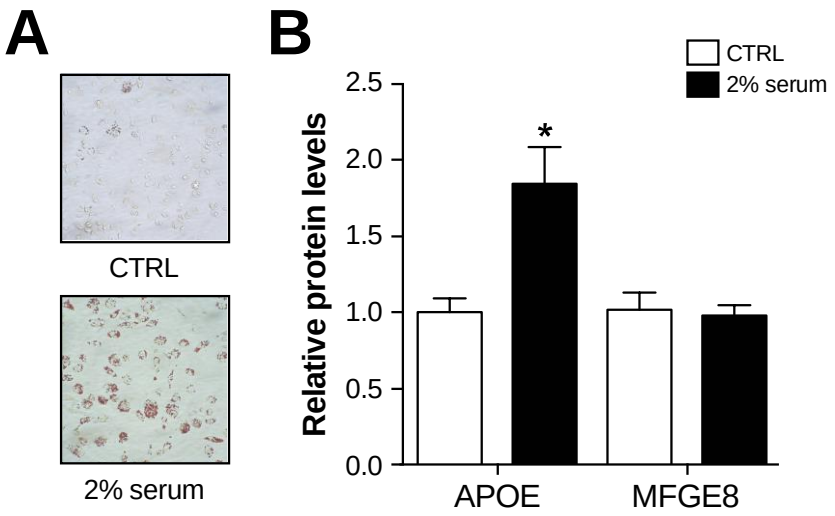


Figure 2.5: In vivo cholesterol loading does not mimic MSRN protein changes observed in vivo. Peritoneal macrophages were treated with and without 2% serum from WTD-fed *Ldlr-/-* mice. Cholesterol loading was confirmed by Oil-red-O staining (*Panel A*) and media APOE and MFGE8 were quantified (*Panel B*). Results are mean ± SEM. *, $p<0.05$ Student's *t*-test; n=4/group.

Macrophages loaded with cholesterol i.e. foam cells, are the hallmarks of atherosclerosis. In macrophages, APOE promotes reverse cholesterol efflux and its expression by macrophages attenuates atherosclerosis even when plasma cholesterol levels are not altered (Fazio et al., 2002). Thus, the suppression of APOE by obesity/IR led us to evaluate whether obesity/IR promotes foam cell formation.

In the '*obesity/IR only*' model, the C57BL/6 mice fed the HFD have low plasma cholesterol levels and hence we were unable to evaluate foam cell formation *in vivo*. Hence, we placed the isolated peritoneal macrophages from these mice in a hypercholesterolemic environment, exposing them to atherogenic lipoproteins from WTD-fed *Ldlr-/-* mice (2% serum) and found that macrophages from obese/IR, HFD-fed mice accumulated more cholesterol than macrophages from lean, LFD-fed mice. Thus, obesity/IR predisposed macrophages to excess cholesterol accumulation and foam cell formation (**Figure 2.4D)**.

Intracellular cholesterol levels in macrophages from both the '*HC only*' model (HCLF diet) and the *HC and obesity/IR'* model (WTD diet) were also measured. Surprisingly, despite being cholesterol loaded in both models, macrophages from '*HC only*' model had lower levels of intracellular cholesterol compared to those from '*HC and obesity/IR'* model (**Figure. 2.4E**). Moreover, aortic lesion area was also reduced in the HCLF diet-fed *Ldlr-/-* mice (**Figure. 2.4F**). However, the caveat with these studies is that the plasma cholesterol levels were significantly lower '*HC only*' model than in the '*HC and obesity/IR'* model, thus further highlighting the problem of dissociating the direct effects of obesity/IR and hypercholesterolemia on foam cell formation and

atherosclerosis progression. We overcame this problem by identifying the regulator of the MSRN and demonstrate that the absence of this regulator attenuates foam cell formation and atherosclerosis progression only in the presence of obesity/IR (see below).

MSRN protein dysregulation promotes macrophage foam cell formation.

Our results show that obesity/IR promotes foam cell formation and one explanation for that could be due to defects in non-MSRN proteins that are involved in cholesterol metabolism. However, mRNA levels of *Abca1*, *Abcg1*, *Lxr*, *Cd36*, *Sra* or *Srebp2* were either unaffected or regulated to oppose cholesterol accumulation in all of the models tested (**Figure 2.6A-B**).

Another explanation for the observed increase in foam cell formation could be due to changes in MSRN proteins such as APOE and C3 that are lowered by obesity/IR in both the HFD-fed *wt* mice and the WTD -fed *Ldlr-/-* mice (**Figure 2.6C**), that have been implicated in lipid metabolism (Barbu et al., 2015; Fazio et al., 2002). Indeed, macrophages from lean 8-week old *Apoe-/-* or *C3-/-* C57BL/6 mice accumulate more cholesterol compared to *wt* macrophages, when exposed to 2% serum from WTD-fed *Ldlr-/-* mice (**Figure 2.6D**). Thus, obesity/IR may promote macrophage cholesterol accumulation by suppressing MSRN proteins such as APOE and C3.

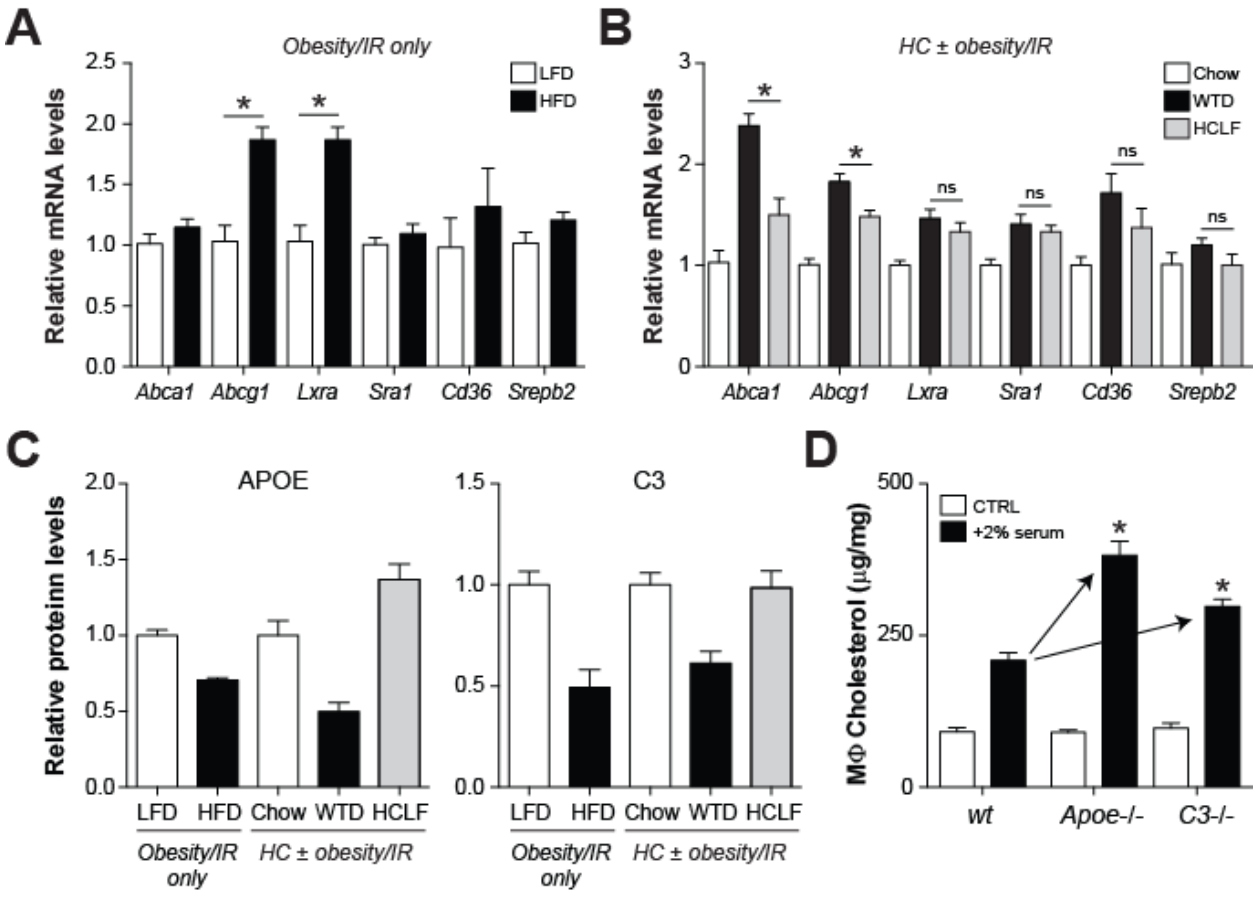


Figure 2.6: MSRN protein dysregulation promotes macrophage foam cell formation. Peritoneal macrophage mRNA levels for several genes involved in cholesterol metabolism in *wt* C57BL/6 mice fed the LFD or HFD ('*obesity/IR only*' model, *Panel A*), or in *Ldlr-/-* mice fed the chow, WTD, or HCLF diet ('*HC ± obesity/IR*' models, *Panel B*). *Panel C*: Peritoneal macrophage media protein levels for APOE and C3 in *wt* C57BL/6 mice fed the LFD or HFD ('*obesity/IR only*' model). *Panel D*: Peritoneal macrophages were isolated from 8-week-old *wt*, *Apoe-/-*, or *C3-/-* C57BL/6 mice fed the LFD. Macrophages were treated were with and without 2% serum from WTD-fed *Ldlr-/-* mice, and cholesterol levels were quantified. Results are mean ± SEM. *, $p<0.05$ (*t*-test). n=5/group.

IFNγ targets MSRN proteins *in vitro* and *in vivo*.

Next, we sought to determine how obesity/IR alter MSRN proteins. We considered the possibility that hyperglycemia and/or hyperinsulinemia was responsible, but found that exposing macrophages to high levels or glucose and insulin, individually

or in combination, had no effect on MSRN proteins such as APOE and MFGE8 (**Figure 2.7A**).

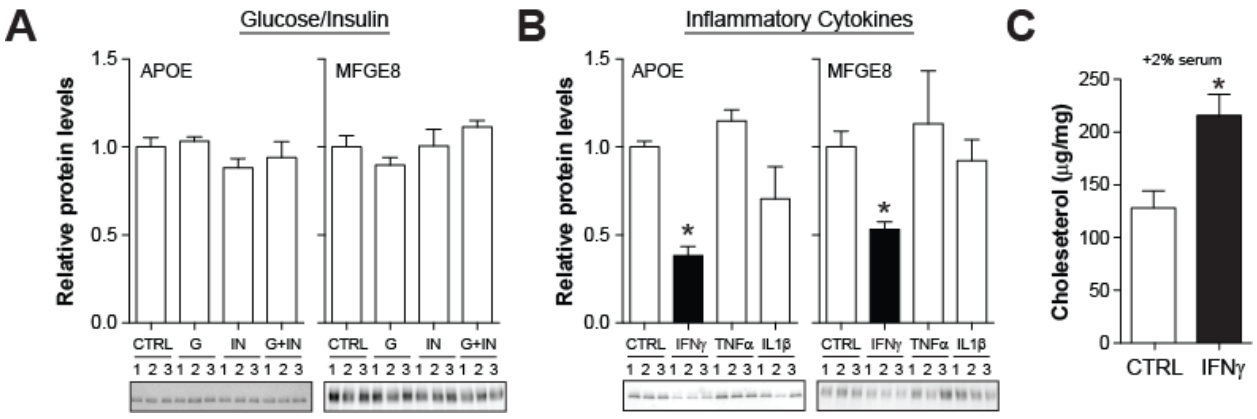


Figure 2.7: IFNγ targets MSRN proteins and promotes macrophage cholesterol accumulation *in vitro*. *Panels A-B*: APOE and MFGE8 levels in peritoneal macrophages treated with vehicle (CTRL), 300 mg/dL glucose (G) or 10nM insulin (I), alone or in combination (G+I), or 12 ng/mL IFNγ, TNFα, or IL1β for 24h. *Panel C*: Cholesterol levels in control and IFNγ-treated macrophages exposed to 2% serum from WTD-fed *Ldlr-/-* mice.

In addition to elevated glucose/insulin levels, patients with T2D have been shown to exhibit low-grade chronic inflammation (Donath and Shoelson, 2011). Treating peritoneal macrophages with various cytokines for their ability to alter MSRN proteins revealed that only IFNγ was able to lower APOE and MFGE8 (**Figure 2.7B**). IFNγ also lowered APOE and MFGE8 in bone marrow-derived macrophages (BMDMs) as well as in human monocyte-derived macrophages (HMDMs) *in vitro* (**Figure 2.8**). In addition, treatment of macrophages with IFNγ followed by exposure to 2% serum from WTD-fed *Ldlr-/-* mice resulted in increased cholesterol accumulation compared to untreated

(**Figure 2.7C**). To confirm that IFNγ signaling is necessary to for MSRN dysregulation, BMDMs from *wt* and *Ifngr1-/-* mice were treated with 12ng/ml IFNγ and showed that in the absence of IFNGR1, IFNγ cannot target MSRN proteins or host defense genes *in vitro* (**Figure 2.9**).

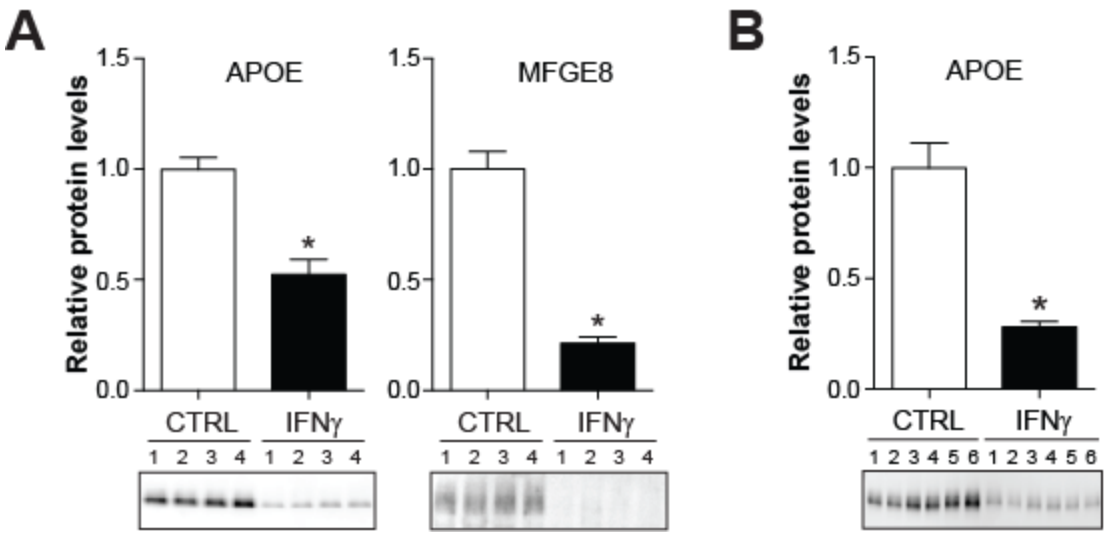


Figure 2.8: IFNγ targets MSRN proteins in many types of macrophages. APOE and/or MFGE8 protein levels in conditioned media collected from murine bone marrow-derived macrophages (*Panel A*) or human monocyte-derived macrophages (*Panel B*) treated with vehicle (CTRL) or IFNγ (12 ng/mL) for 24h. Results are mean ± SEM. *, $p<0.05$ Student's *t*-test. n=4-6/group.

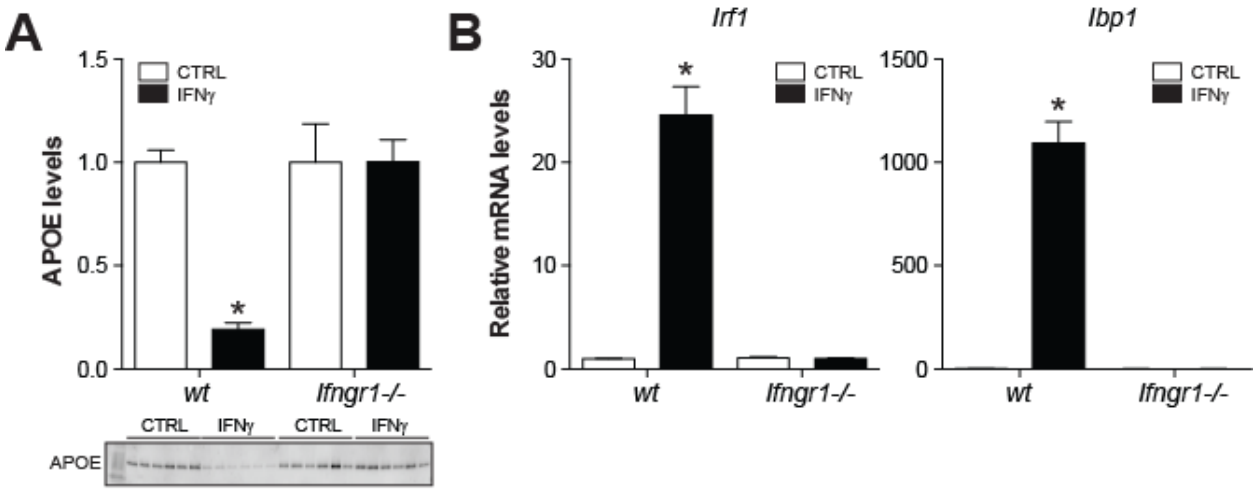


Figure 2.9: IFNGR1 is required for IFNγ to target MSRN proteins and its target genes *in vitro*. Wild-type (*wt*) and *Ifngr1-/-* macrophages were treated with vehicle (CTRL) or 12ng/mL IFNγ. *Panel A*: Media APOE levels. *Panel B*: mRNA levels of IFNγ-target genes. Results are mean ± SEM. *, $p<0.05$ Student's *t*-test. n=6/group.

The critical next step was to determine whether IFNγ could alter MSRN proteins in murine artery wall macrophages (MAMs) *in vivo*. This is important because macrophage biology is largely driven by tissue-specific microenvironments (Gordon and Taylor, 2005). To do so, we developed a new approach to isolate MAMs from atherosclerotic *Ldlr-/-* mice and interrogate their responses to IFNγ by proteomics (**Figure 2.10A**). Briefly, *Ldlr-/-* mice were placed on a WTD for 16 weeks to allow MAMs to accumulate. At this time, mice were injected with IFNγ (IP, 25 μg/Kg) or vehicle control, following which MAMs were isolated**.** Lesions from 10-12 *Ldlr-/-* mice were pooled, digested and the isolated cells were purified using CD11b magnetic beads, yielding ~50,000 MAMs in the elute (**Figure 2.10B)**. The purified MAMs were plated and the conditioned media collected 24h post-plating. MSRN proteins in the conditioned media were quantified by proteomics (**Figure 2.10C-D)**.

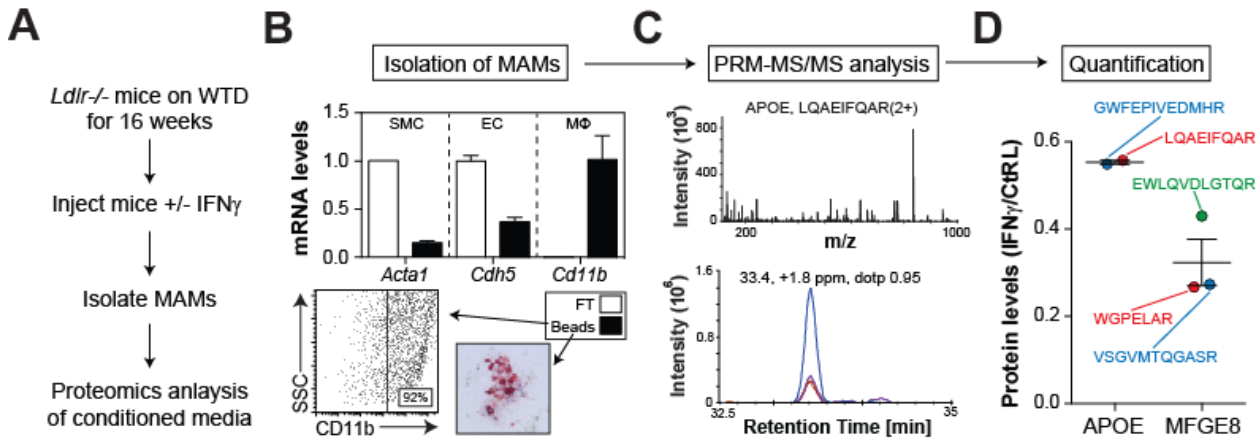


Figure 2.10: IFNγ targets MSRN proteins in artery wall macrophages. *Panel A*: *Ldlr-/-* mice were fed a WTD for 16 weeks, injected with vehicle or IFNγ, and murine aortic macrophages (MAMs) were isolated. *Panel B*: MAM purity was assessed by qRT-PCR for smooth muscle cells (SMC), endothelial cells (EC), and macrophage (MΦ) markers in anti-CD11b bound (beads) and unbound (FT) cells, flow cytometry, and Oil-red-O staining. *Panel C*: Representative MS/MS spectrum and ion chromatograms from parallel reaction monitoring. *Panel D*: Relative quantification of APOE and MFGE8 for all peptides in IFNγ-injected versus control mice. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3-5/group.

Using parallel reaction monitoring (PRM) method of mass spectrometry, (Hoofnagle et al., 2012), our collaborator, Dr. Tomas Vaisar, at the University of Washington, Seattle, was able to quantify several MSRN proteins in small numbers of MAMs isolated from *Ldlr-/-* mice. After validating that this approach could reliably quantify APOE in a 50,000 macrophages (**Figure 2.11**), we analyzed the abundance of APOE and MFGE8 in MAM conditioned media. Our results showed that IFNγ lowered both APOE and MFGE8 in MAMs *in vivo* (**Figure 2.10C-D**), suggesting that changes in circulating, peripheral IFNγ can target murine artery wall macrophages and alter their MSRN proteins *in vivo*.

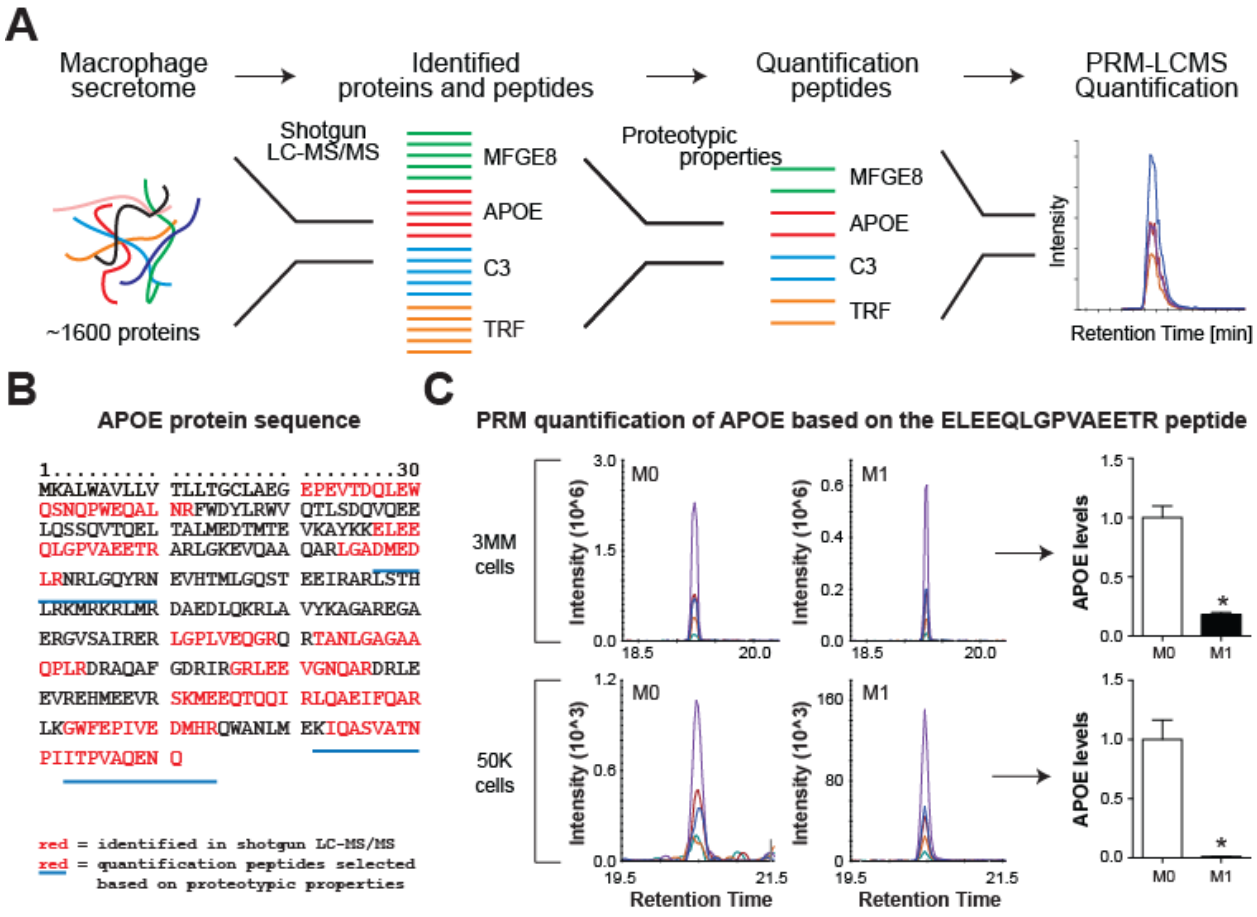


Figure 2.11: Parallel reaction monitoring quantifies proteins in limited numbers of macrophages. *Panel A*: Development of a targeted proteomics assay for quantification of MSRN proteins. MSRN proteins and identified peptides were selected from shotgun proteomics analysis and further evaluated for proteotypic properties using public resources. Best peptides (up to 5 per protein where available) were selected and further investigated using Parallel Reaction Monitoring LCMS in samples from unstimulated (M0) or classically activated (M1) BMDMs. Two conditioned were used: 3 million macrophages were cultured and proteins in the conditioned media were precipitated as previously reported (Becker et al., 2010) and 0.2 µg was injected, or 50,000 macrophages were cultured and conditioned media were analyzed directly. *Panel A*: Workflow diagram. *Panel B*: Protein sequence of APOE indicating peptides selected for PRM quantification. *Panel C*: Quantification of APOE based on the ELEEQLGPVAEETR peptide in 3million or 50,000 M0 and M1 macrophages.

Obesity/IR-induced MSRN protein dysregulation and macrophage cholesterol accumulation are corrected in obese/IR *Ifngr1-/-* mice.

Based on our *in vitro* data that IFNγ lowered MSRN proteins and enhanced cholesterol loading, we hypothesized that IFNγ was required for obesity/IR to target the MSRN and promote cholesterol accumulation *in vivo*. To test this, we first determined if IFNγ levels were raised in the '*obesity/IR only*' model. Since plasma IFNγ levels were undetectable, we quantified IFNγ production by activated splenic T cells, an abundant, but not exclusive source of this cytokine *in vivo*. IFNγ secreted by T cells in the presence of obesity/IR was higher than that compared to *wt* mice (**Figure 2.12**).

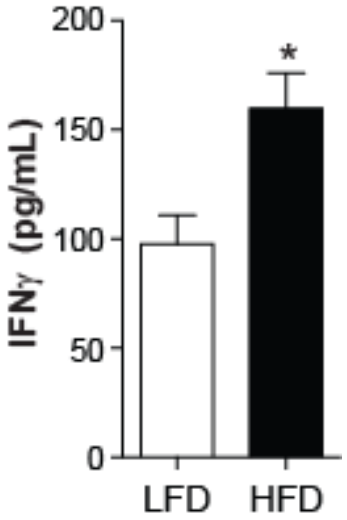


Figure 2.12: IFNγ production by *wt* splenic T-cells in the '*obesity/IR only*' model. *Wt* mice were fed a LFD or HFD for 9 weeks. Splenic T cells were isolated, activated with anti-CD3 and anti-CD28, and conditioned media was collected and analyzed for IFNγ by ELISA. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=4-10/group.

Next, to determine whether IFNγ is necessary for obesity/IR to alter MSRN proteins and promote macrophage cholesterol accumulation in vivo, we put *wt* and *Ifngr1-/-* mice on LFD and HFD diets, i.e. '*obesity/IR only*' model. Deleting *Ifngr1* did not correct metabolic parameters in obese/IR mice (**Figure 2.13A**), which, in accordance with other studies (Rocha et al., 2008), suggests that IFNγ does not appreciably contribute to insulin resistance.

In contrast, deletion of *Ifngr1* resulted in the normalization of dysregulated MSRN proteins observed in *wt* obese/IR mice (**Figure 2.13B, Table 2.1**) as well as blocked the ability of obesity/IR to promote macrophage cholesterol accumulation *in vitro* following treatment with 2% serum from WTD-fed *Ldlr-/-* mice (**Figure 2.13C**). This reduced cholesterol accumulation in macrophages from obese/IR *Ifngr1-/-* mice could not however, be explained due to changes in the expression of cholesterol metabolism genes-*Abca1*, *Abcg1*, *Lxra*, *Sra1*, *CD36* and *Srebp2* (**Figure 2.13D**).

Myeloid cell-deletion of *Ifngr1* attenuates atherosclerosis in *Ldlr-/-* mice only in the presence of obesity/IR.

Our findings suggest that obesity/IR was required to induce IFNγ and pro-atherogenic changes to macrophages. Additionally, the effects of IFNγ on the progression of atherosclerosis has been well established in numerous mouse studies (Ramji and Davies, 2015). Thus, taken together, we hypothesized that obesity/IR results in the induction of IFNγ production, which in turn, alters MSRN proteins to promote macrophage cholesterol accumulation and atherogenesis in the presence of hypercholesterolemia.

To test our hypothesis, we transplanted lethally irradiated *Ldlr-/-* mice with bone marrow from either *wt* or *Ifngr1-/-* mice and fed them a chow, WTD ('*HC and obesity/IR'* model) or HCLF ('*HC only'* model) diet (**Figure 2.14)**. This approach allowed us to ascertain the physiological implications of obesity/IR and myeloid-specific IFNγ signaling on atherosclerosis *in vivo*.

A

	HFD	
Metabolic measure	***wt***	***Ifngr1-/-***
Body weight (g)	35 ± 4	33.1 ± 0.4
Glucose (mg/dL)	159 ± 16	157 ± 4
Insulin (nM)	1.9 ± 0.4	1.5 ± 0.2
Plasma cholesterol (mg/dL)	136 ± 5	108 ± 11

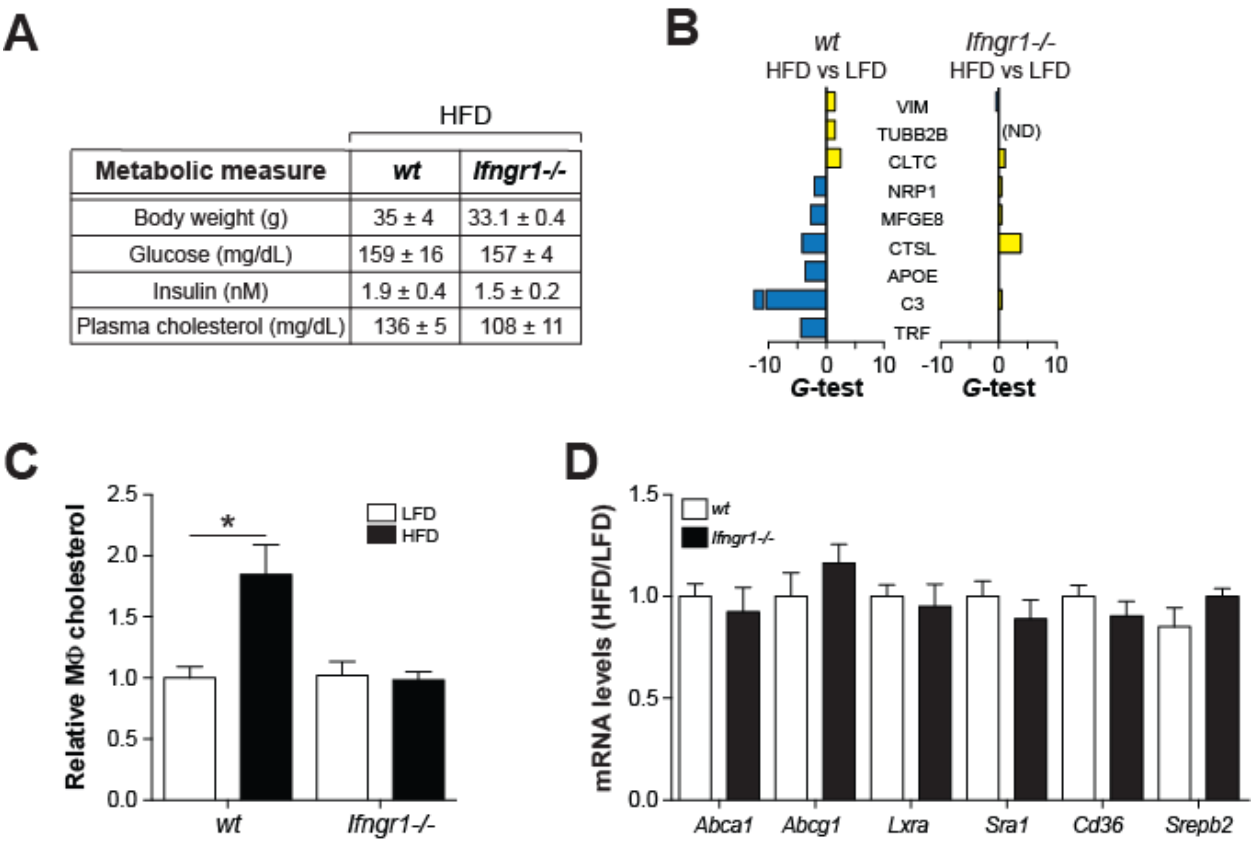


Figure 2.13: Deleting Ifngr1 corrects MSRN proteins and macrophage cholesterol accumulation in the '*obesity/IR only*' model. *Panels A-G*: *Wt* and *Ifngr1-/-* mice were fed a LFD or HFD for 9 weeks. *Panel A*: IFNγ production by splenic T-cells in *wt* mice. *Panel B*: Metabolic parameters. *Panels C-G*: Peritoneal macrophage MSRN protein levels (*Panel C*), cholesterol levels following treatment with 2% serum from WTD-fed *Ldlr-/-* mice (*Panel D*), cholesterol metabolism gene levels (*Panel E*). Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=4-10/group.

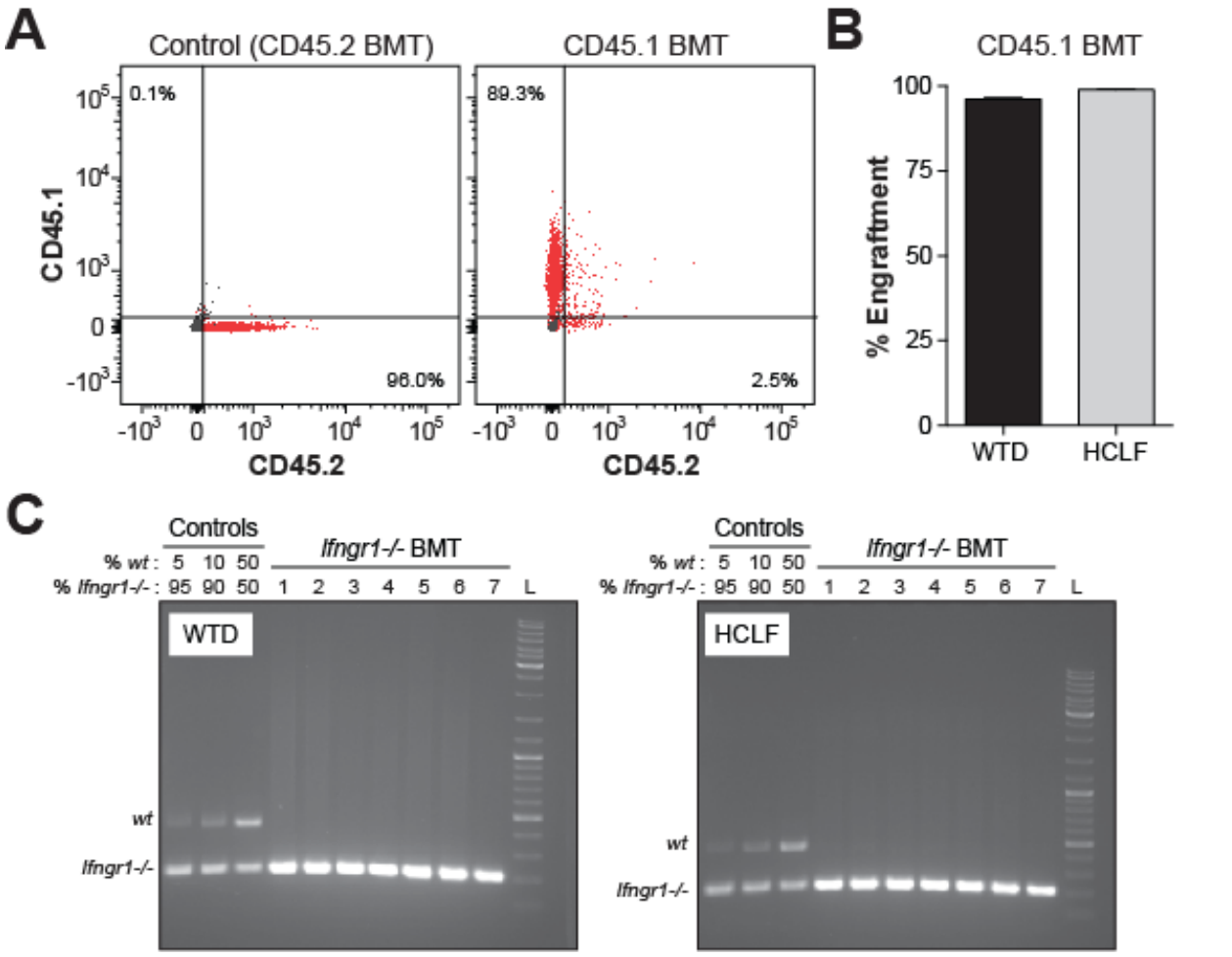


Figure 2.14: Bone marrow engraftment. *Panels A-B*: Engraftments for *wt* bone marrow transplants into *Ldlr-/-* mice recipient mice were assessed by flow cytometric analysis of the ratio of CD45.1 (donor) to CD45.2 (recipient) positive bone marrow cells. *Panel C*: Engraftments for *Ifgnr1-/-* bone marrow transplants into *Ldlr-/-* mice recipient mice were **Figure 2.14 continued:** assessed by PCR analysis of the ratio of *Ifngr1-/-* (donor) and *wt* (recipient) in comparison to pre-defined mixtures of bone marrow from *wt* and *Ifngr1-/-* mice. Seven mice per dietary condition are shown as examples. All engraftments were judged to be >95%. Results are mean ± SEM. n=11-12/group.

Similar to the 'obesity/IR only' model (see Figure 2.12A), splenic T cells from the WTD-fed, but not the HCLF-fed *wt* bone marrow-transplanted *Ldlr-/-* mice had elevated IFNγ production (**Figure 2.15**). Importantly, deletion of *Ifngr1* in bone marrow-derived

cells did not correct metabolic parameters of the mice fed either diet (**Figure 2.16A**). In contrast, deletion of *Ifngr1* restored 5/9 MSRN proteins in the '*HC and obesity/IR*' model but had no effect on these proteins in the '*HC only*' model (**Figure 2.16B-C, Table 2.4**). Additionally, the absence of *Ifngr1* also lowered macrophage cholesterol levels in WTD-fed *Ldlr-/-* mice, but had no effect in the HCLF diet-fed mice (**Figure 2.16D**). The decrease in foam cell formation in the WTD-fed mice could not be explained due to changes in mRNA levels of *Abca1*, *Abcg1*, *Lxra*, *Sra1*, *CD36* and *Srebp2*, all of which were unchanged in *Ifngr1*-deficient macrophages compared to *wt* (**Figure 2.16E**).

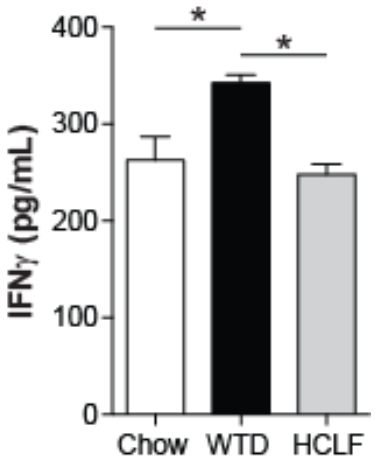


Figure 2.15: IFNγ production by splenic T-cells in *wt* bone marrow-transplanted mice. *Ldlr-/-* mice transplanted with *wt* bone marrow cells were fed a chow, WTD, or HCLF diet for up to 15 weeks. Splenic T cells were isolated, activated and conditioned media was collected and analyzed for IFNγ by ELISA. Results are mean ±SEM. *, $p<0.05$ (*t*-test); n=3-12/group.

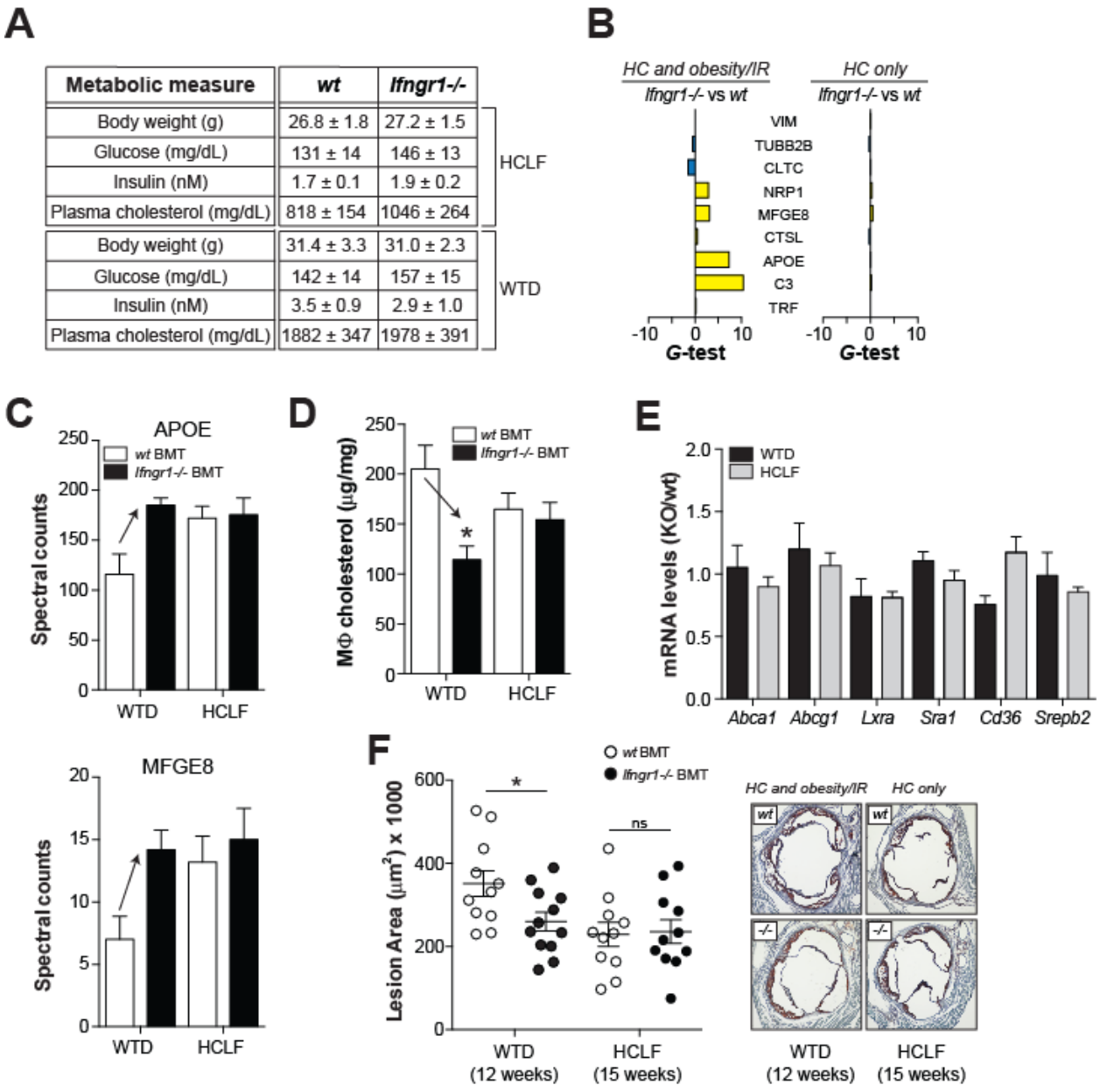


Figure 2.16: Macrophage IFNGR1 is required for obesity/IR to target the MSRN and promote foam cell formation and atherogenesis. *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow cells were fed a chow, WTD, or HCLF diet for up to 15 weeks. *Panel A*: Metabolic parameters. *Panels B-E*: Peritoneal macrophage MSRN protein levels (*Panels B-C*), cholesterol levels (*Panel D*), cholesterol metabolism gene levels (*Panel E*). *Panel F*: Aortic root lesion area and representative images. Results are mean ±SEM. *, $p<0.05$ (*t*-test); n=3-12/group.

HC +/- Obesity/IR Model (Bone marrow transplantation)						
Protein annotations			*Ldlr-/-* mice WTD (*Ifngr1-/-* vs. *wt* BMT)		*Ldlr-/-* mice HCLF (*Ifngr1-/-* vs. *wt* BMT)	
Uniprot	Entrez	Protein	G-test (-/- : *wt*)	t-test	G-test (-/- : *wt*)	t-test
P01027	12266	C3	14.10	0.0190	0.29	0.6344
Q921I1	22041	TRF	0.19	0.1629	0.00	0.9837
P06797	13039	CTSL	0.50	0.1482	-0.25	0.1226
P08226	11816	APOE	7.37	0.0448	0.03	0.8749
P21956	17304	MFGE8	3.07	0.0073	0.52	0.2275
P97333	18186	NRP1	2.90	0.0030	0.38	0.1316
P20152	22352	VIM	0.00	0.9569	0.04	0.6143
B2RSN3	73710	TUBB2B	-0.52	0.0317	-0.24	0.1028
Q68FD5	67300	CLTC	-1.50	0.0240	0.02	0.6031

Table 2.4: Shotgun proteomic analysis of elicited peritoneal macrophages from *HC* ± *obesity/IR*' model (bone marrow transplantation). Proteomics analysis of the conditioned media collected from elicited peritoneal macrophages isolated from *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow fed the WTD or HCLF diet. Proteomics data were analyzed by the G-test (G-statistic) and t-test (p-value). Differentially abundant proteins are shaded; red = up-regulated in 1st sample relative to the 2nd, green = down-regulated in the 1st sample relative to the 2nd. ND = not detected. n=3-5 mice/group.

Importantly, the deletion of *Ifngr1* in myeloid cells resulted in a decrease in aortic root lesion area only in *Ldlr-/-* fed the WTD for 12 weeks, i.e. in the '*HC and obesity/IR*' model (**Figure 2.16F, Figure 2.17**). This reduction could not be explained by changes in metabolic parameters (see Figure 2.16A) or in plasma lipoprotein distribution (**Figure 2.18**). In contrast, lesion area was not lowered in mice fed the HCLF diet for 15 weeks (to compensate for delayed lesion formation) (**Figure 2.16F, Figure 2.17**).

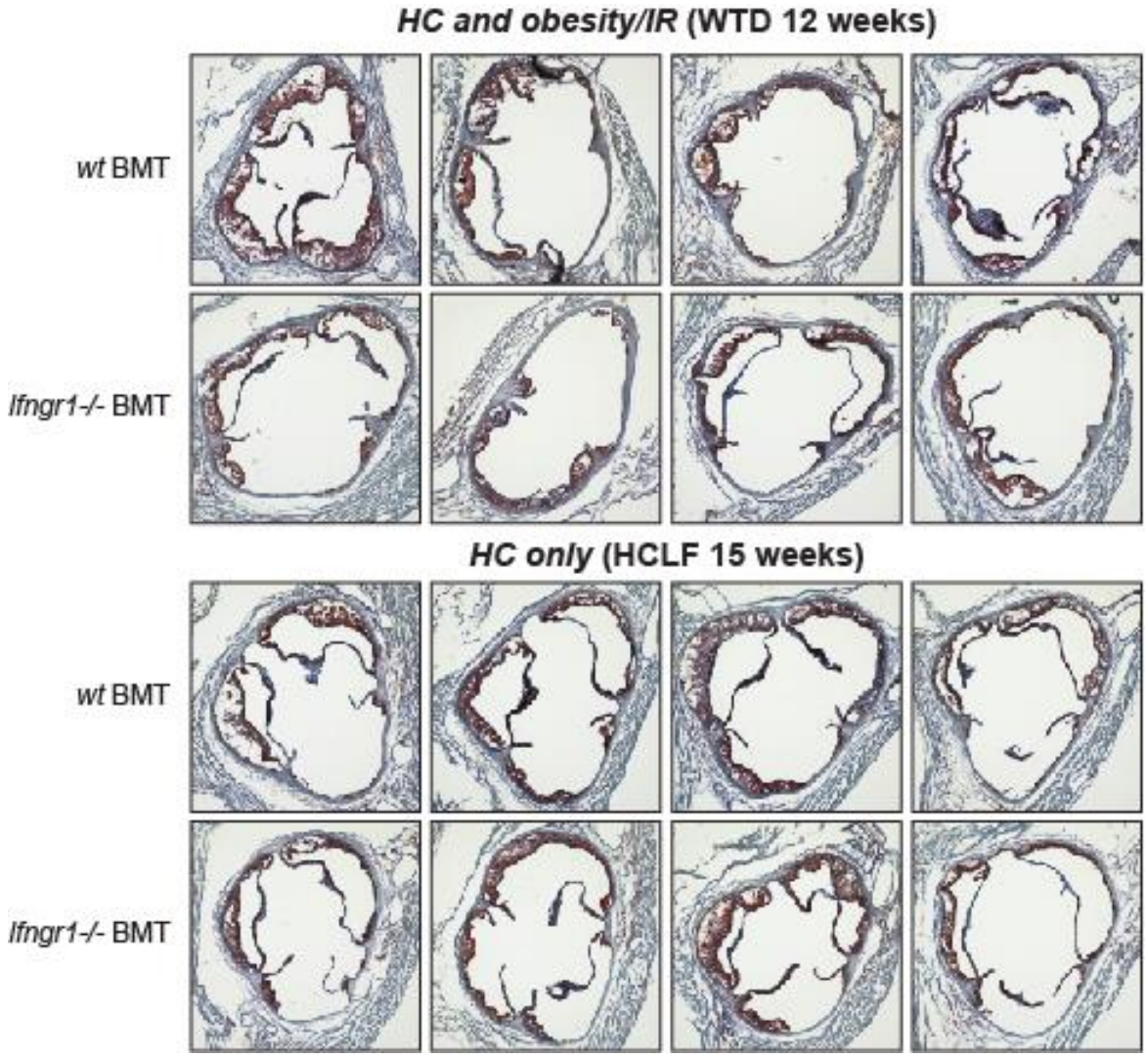


Figure 2.17: Representative images of aortic root lesions from *wt* and *Ifngr1-/-* bone marrow transplants into *Ldlr-/-* mice. *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow cells were fed a WTD or HCLF diet for up to 15 weeks. Cross-sections of the aortic root were stained with Oil-red-O, counterstained with hematoxylin and fast green, and lesion area was quantified. Four representative images are shown per group.

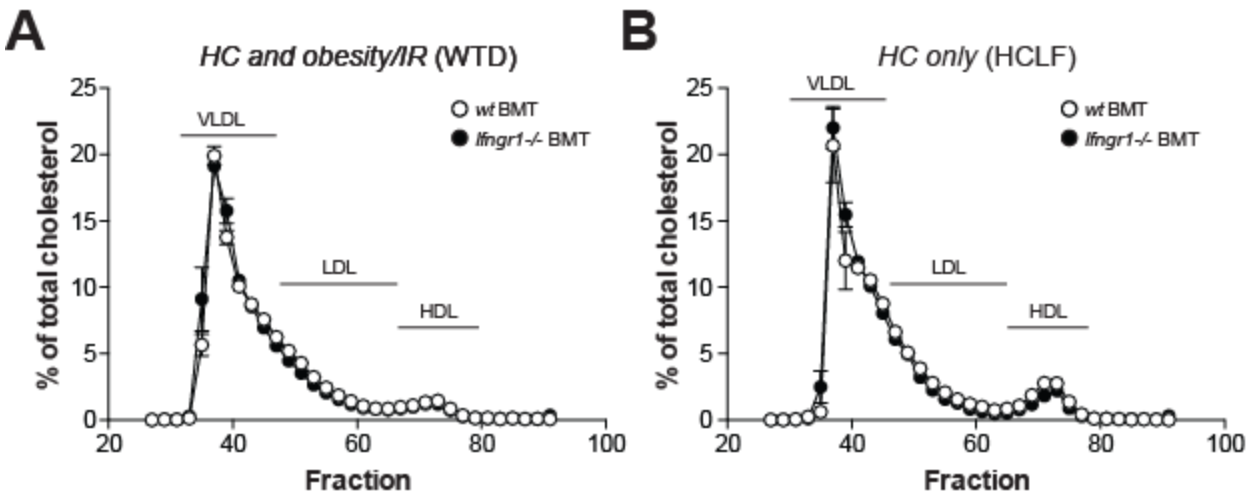


Figure 2.18: Myeloid cell deletion of *Ifngr1-/-* does not impact plasma lipoprotein profiles. *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow cells were fed a WTD or HCLF diet for up to 15 weeks. Plasma (100 µL) was fractionated by gel filtration using two Superose-6 columns in tandem, and cholesterol levels in each fraction were quantified using the Amplex Red Cholesterol Assay kit (Invitrogen). Lipoprotein cholesterol levels in WTD-fed mice (*Panel A*) and HCLF diet-fed mice (*Panel B*). Results are mean ± SEM. n=5/group.

Finally, in the 'HC and obesity/IR' model, plasma insulin levels but not plasma cholesterol levels positively correlated to aortic root lesion size of the *Ldlr-/-* mice (**Figure 2.19A-B**) (Gruen et al., 2006). Importantly, the deletion of *Ifngr1* abolished this relationship (**Figure 2.19A-B**), without any changes in the metabolic parameters (see Figure 2.16A).

A

	HC and obesity/IR			
	wt BMT		*Ifngr1-/-* BMT	
Metabolic measure	**R^2**	***p*-value**	**R^2**	***p*-value**
Body weight	0.06	0.469	0.19	0.158
Fasting glucose	0.16	0.225	0.10	0.323
Fasting insulin	0.64	0.005	0.01	0.890
Plasma cholesterol	0.04	0.549	0.08	0.372

B

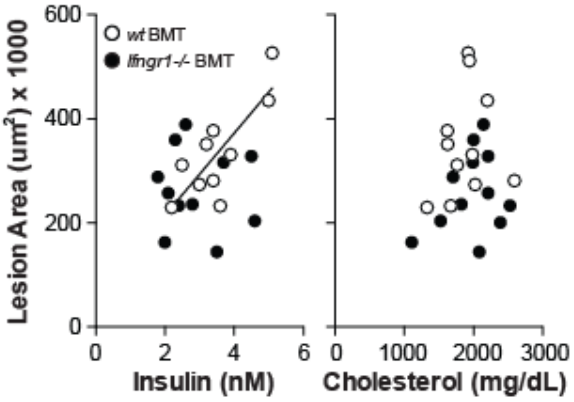


Figure 2.19: Statistical correlation analysis between *wt* and *Ifngr1-/-* BMT mice. *Ldlr-/-* mice transplanted with *wt* or *Ifngr1-/-* bone marrow cells were fed a chow, WTD, or HCLF diet for up to 15 weeks. *Panels A-B*: Relationships between metabolic parameters and aortic root lesion area. Results are mean ±SEM. *, $p<0.05$ (*t*-test); n=3-12/group.

Overall, our data demonstrates that abrogation of macrophage IFNγ signaling protects against obesity/IR-associated atherosclerosis progression, thereby providing physiological evidence of the impact of macrophage dysregulation *in vivo* and identifies obesity/IR-induced IFNγ as the driver of this macrophage dysfunction.

Discussion

Patients with T2D have a 2-4 fold increased risk for atherosclerosis compared to non-diabetics, which accounts for ~70% morbidity and mortality in these patients (Beckman et al., 2002; Gore et al., 2015; Haffner et al., 1998; Hayward et al., 2015). Despite these staggering statistics, the mechanisms explaining this increased risk are poorly understood, partly because the diets that induce obesity/IR in *Ldlr-/-* and *Apoe-/-* mice, the most common murine models of atherosclerosis, also elevate plasma cholesterol. Thus, it has been challenging to dissociate direct effects of T2D on the

progression of atherosclerosis, which is important because increased cardiovascular events in diabetic patients cannot be fully explained by hypercholesterolemia alone (Costa et al., 2006).

To overcome this challenge, we used a combination of genetic (*wt* and *Ldlr-/-* mice) and dietary (HFD, WTD, HCLF diets) approaches to study macrophages from mice with obesity/IR only, HC & obesity/IR, or HC only. This approach has allowed us to identify an IFNγ-macrophage-MSRN pathway that promotes foam cell formation and atherogenesis, only in the context of obesity/IR.

Three lines of evidence support the specificity of this pathway for obesity/IR. First, we demonstrated that obesity/IR is both necessary and sufficient to induce IFNγ production by T cells. Second, abrogation of IFNγ signaling in macrophages (by myeloid-specific deletion of *Ifngr1* using bone marrow transplantation) resulted in attenuation of atherosclerotic lesion size in hypercholesterolemic *Ldlr-/-* mice only in the presence of obesity/IR. Third, blocking macrophage IFNγ signaling abolished the strong relationship between hyperinsulinemia and aortic root lesion area without affecting the extent of the metabolic syndrome in these mice.

Together, these findings suggest that some of the mechanisms that regulate the progression of atherosclerosis in the presence and absence of obesity/IR are distinct, and identify IFNγ as the driver of T2D-associated atherosclerosis. Therapies targeting pro-inflammatory cytokines in patients with increased risk of cardiovascular diseases are currently being tested (Ridker, 2016) and our work suggests that anti-IFNγ therapeutics might be particularly beneficial for lowering risk in patients with T2D.

At a molecular level, we have demonstrated that IFNγ suppressed specific anti-atherogenic MSRN proteins (i.e. APOE and C3), which predispose macrophages to increased cholesterol accumulation. Macrophages from obese/IR mice also had reduced APOE and C3 levels, but did not exhibit defects in other cholesterol metabolism genes such as *Abca1*, *Abcg1*, *Lxra*, *Sra1*, *CD36* and *Srebp2*. Similarly, the absence of *Ifngr1* in obese/IR attenuated foam cell formation and restored APOE and C3 levels without affecting any of the other cholesterol metabolism genes. Thus, suppression of APOE and C3 and perhaps other MSRN proteins, represents a key mechanism by which IFNγ and obesity/IR may promote foam cell formation.

However, IFNγ-induced changes to MSRN proteins are insufficient to cause foam cell formation (or atherogenesis) in the absence of hypercholesterolemia. Instead, these changes result in a molecular susceptibility that, in the presence of hypercholesterolemia, increases macrophage cholesterol accumulation, a central event in atherogenesis. From this perspective, obesity/IR can be conceptualized as a perturbation that “sensitizes” macrophages to atherogenic lipoproteins, which may help to explain why T2D patients generally require more aggressive cholesterol lowering to achieve therapeutic benefit (Banach et al., 2016; Hoe and Hegele, 2015).

Importantly, deleting *Ifngr1* did not affect metabolic parameters in normocholesterolemic (*wt*) and hypercholesterolemic (*Ldlr-/-*) mice, which agrees with previous studies using *Ifng-/-* mice (Rocha et al., 2008). The uncoupling of metabolic and pro-atherogenic functions of IFNγ might help to explain the poorly understood relationship between metabolic dysfunction and atherosclerotic risk in T2D patients. While elevated HbA1c levels are an excellent predictor of atherosclerotic risk,

therapeutic lowering of HbA1c does not necessarily alleviate this risk (Hayward et al., 2015). These data suggest that T2D induce factor(s) that promote atherogenesis, but these factors(s) are not necessarily corrected by insulin sensitizing interventions. Recent studies have shown that systemic and adipose tissue inflammation persists following bariatric surgery despite correction of hyperglycemia and insulin sensitivity (Kratz et al., 2016). Based on these studies and our work, we speculate that IFNγ might be one of these factors; however additional studies are needed to confirm this hypothesis.

IFNγ has myriad functions, one of the most important being host defense. Future studies aimed at dissecting the specificity of the IFNγ-macrophage-MSRN pathway are crucial for the development of therapeutic to alleviate risk of cardiovascular diseases without compromising immune functions in T2D patients.

CHAPTER THREE: IFNγ TARGETS THE MACROPHAGE-STEROL RESPONSIVE NETWORK (MSRN) THROUGH A HOST DEFENSE-INDEPENDENT MECHANISM

Introduction

IFNγ has been known historically as the master regulator of atherosclerosis and has been studied extensively in order to understand how it contributes to the development of cardiovascular diseases (Boshuizen and de Winther, 2015; McLaren and Ramji, 2009; Ramji and Davies, 2015; Voloshyna et al., 2014; Yu et al., 2015). IFNγ producing immune cells are prominent in both human and mouse atheromas and it impacts all cell types in the artery wall to contribute to atherogenesis (Libby et al., 2013). This apart, IFNγ has been shown to exert anti-atherogenic functions as well (Harvey and Ramji, 2005). Amongst all the different pro-inflammatory cytokines present within the plaques, IFNγ plays a pivotal role in the progression of atherosclerosis. This chapter provides a review of the role of IFNγ in promoting foam cell formation and atherogenesis and highlights the different mechanisms by which it impacts macrophage functions in lesion development. Previously, we have identified IFNγ as the driver of obesity/IR-induced MSRN dysregulation which results in increased foam cell formation and atherosclerosis. Here, we provide evidence for a host defense-independent mechanism through which IFNγ targets the macrophage MSRN. An understanding of this mechanism may lead to potential therapeutics that can reduce atherosclerotic burden without compromising other vital functions of IFNγ.

Initially identified due to its role in immune regulation of host defense, IFNγ has emerged over the years to have myriad functions in various different cell types such as regulation of antigen presentation and immune cell activation, secretion of chemokines, cell proliferation and initiation of programmed cell death (McLaren and Ramji, 2009). IFNγ is a pro-inflammatory cytokine involved in both innate and adaptive immune responses and secreted by many different cell types like monocytes, macrophages, T cells, natural killer (NK) cells and dendritic cells (DC) (Ilhan and Kalkanli, 2015; McLaren and Ramji, 2009; Moore and Tabas, 2011; Tabas and Lichtman, 2017).

IFNγ signaling through its canonical pathway involves the ligand-binding IRNGR1 receptor that heterodimerizes with tyrosine-kinase IFNGR2 receptor, upon interaction with IFNγ resulting in a cascade of phosphorylation events for signal transduction. IFNGR2 receptor-associated proteins such as Jak1, Jak2 and Stat1 are the key signal transducers in this canonical pathway. Phosphorylation of STAT1 causes dissociation with IFNGR2 and results in subsequent dimerization and translocation of this transcription factor into the nucleus for the induction of IFNγ-target genes (Majoros et al., 2017). Studies have demonstrated the STAT1-independent effects of IFNγ as well, where it functions through mechanisms involving extra-cellular regulated kinase (ERK) and phosphatidylinositol-3 (PI-3) kinase (Ramana et al., 2002).

Within the atheroma, IFNγ has been shown to exert both pro- and anti-atherogenic functions. It encourages atherogenesis by inducing increased expression of adhesion molecules like VCAM-1 and ICAM-1 that aide in the tethering and extravasation of monocytes from the blood into the sub-endothelial space of the artery wall (Boshuizen and de Winther, 2015). Within this space, monocytes differentiate into

macrophages that ingest the lipid retained in the artery wall to form ‘foam cells’, a hallmark of atherosclerosis.

IFNγ has been shown to contribute to both, cholesterol uptake and efflux in macrophages. Cholesterol uptake occurs through scavenger receptors (SR) on macrophages and IFNγ was found to enhance SR-A receptor expression in THP-1 cells, a monocyte cell line, and promote uptake of acetylated LDL in these cells (Grewal et al., 2001; Reiss et al., 2004). On the other hand, studies have demonstrated that IFNγ plays an important role in reverse cholesterol transport, causing a downregulation of ABCA1 as well as suppressing APOE synthesis in macrophages (Brand et al., 1993; Panousis and Zuckerman, 2000). Moreover, IFNγ promotes foam cell formation by increasing ACAT expression in macrophages (Panousis and Zuckerman, 2000). In contrast, by inhibiting lipoprotein lipase (LPL) expression in macrophages and thereby preventing the breakdown of VLDL and increased uptake of LDL, IFNγ contributes to anti-atherogenic functions of macrophages (Mead and Ramji, 2002).

Experimentally, exogenous injection of IFNγ promotes atherosclerosis in *Apoe-/-* mice (Whitman et al., 2000) and conversely, depletion of IFNγ (*Ifng-/-*) in mice reduces atherosclerosis (Buono et al., 2003; Whitman et al., 2002). Similarly, abrogating IFNγ signaling by eliminating its ligand-binding receptor (*Ifngr1-/-*) or injecting a soluble decoy receptor has been shown to reduce the degree of atherosclerosis (Gupta et al., 1997; Koga et al., 2007a; Koga et al., 2007b).

However, in addition to promoting atherogenesis, IFNγ also plays a vital role in regulating host defense. Mice treated with IFNγ neutralizing antibodies or that are deficient in *Ifng* or *Ifngr* are susceptible to a variety of bacterial infections (Dalton et al.,

1993; Ismail et al., 2002; Kamijo et al., 1993; Lee et al., 2013; Shtrichman and Samuel, 2001), and some viruses as well (Cantin et al., 1999; Chesler and Reiss, 2002; Huang et al., 1993). Moreover, humans with mutations that interfere with IFNγ signaling or its production are susceptible to Mycobacteria, Salmonella and other bacterial infections (Al-Muhsen and Casanova, 2008; Zhang et al., 2008).

Due to its essential role in regulating host innate immunity, IFNγ is a poor therapeutic target for the treatment and management of atherosclerosis. However, despite affecting many cell types, IFNγ's action on macrophages may be central to its pro-atherogenic and anti-bacterial properties. *In vitro*, IFNγ promotes a pro-inflammatory M1 phenotype, increases expression of scavenger receptors ACAT1 and matrix metalloproteases (MMPs) and decreases ABCA1 and APOE levels, phenotypes that have been shown to promote atherosclerosis (Boshuizen and de Winther, 2015; Voloshyna et al., 2014; Yu et al., 2015). Anti-bacterial effects of IFNγ in macrophages include (i) induction of NO and ROS, (ii) induction of small GTPases that influence phagosomes, autophagy and anti-microbial peptide release, and (ii) depletion of tryptophan needed for bacterial growth (Ismail et al., 2002; Kaufmann and Dorhoi, 2016; Shtrichman and Samuel, 2001). Thus, IFNγ can induce both protective and harmful responses in macrophages, depending on the context in which they are viewed.

Despite being extensively studied, the dietary conditions supporting the induction of IFNγ, the molecular changes it induces *in vivo* and the cellular target(s) that mediates its pro-atherogenic effects are incompletely understood. Patients with type 2 diabetes have an increased risk for and suffer from a high mortality rate due to atherosclerosis

and other cardiovascular complications. However, the mechanisms underlying this increased atherosclerotic risk in patients with type 2 diabetes are also unknown.

We have identified IFNγ to be the driver of this increased atherosclerotic susceptibility in obese/IR mice. We have shown that obesity/IR-induces IFNγ that specifically targets macrophages to produce dysregulation of the MSRN, a network causally implicated in atherosclerosis and promotes macrophage cholesterol accumulation. Here, we provide evidence that the pro-atherosclerotic and anti-bacterial properties of IFNγ are mechanistically distinct. We show that exposing macrophages to 'metabolic disease-appropriate' doses of IFNγ in vitro targets MSRN proteins, but does not induce canonical IFNγ signaling, IFNγ-responsive genes, or bacterial killing, all of which require higher doses. Additionally, we show that deleting/attenuating components of canonical IFNγ signaling blocks IFNγ-enhanced bacterial killing by macrophages, but do not block IFNγ action on the MSRN. Moreover, ablating *Ifngr1* in obese/IR mice corrects MSRN and attenuates macrophage cholesterol accumulation, but does not affect genes induced by canonical IFNγ signaling.

Delineating the disparate mechanisms by which IFNγ activates macrophage bacterial killing and targets the MSRN, could provide the rationale to antagonize its action on atherosclerosis without interfering with host defense. Thus, this work aims at understanding the interplay between IFNγ, macrophages, obesity/IR and atherosclerosis to maximize strategies to treat cardiovascular disease without predisposing patients to opportunistic infections.

Materials and Methods

Mice: All animal studies were approved by the University of Chicago IACUC (ACUP#72209). Wild-type (CD45.1 or CD45.2), *Ldlr-/-*, and *Ifngr1-/-* mice on the C57BL/6 background are from Jackson Labs. For DIO studies, wild-type (*wt*) and *Ifgnr1-/-* mice were placed on a low-fat (20:50503, PicoLab) or 60% high-fat diet (D12492, Research Diets Inc.) for 9 weeks.

Isolation of macrophages: Bone marrow-derived macrophage isolation and activation –Bone marrow cells were isolated from the femur and tibia of wildtype C57/Bl6 wildtype mice (Jackson Labs) and pooled. Cells were plated in Roswell Park Memorial Institute medium (RPMI) containing 10% FBS and 30% L-conditioned media. Media was changed on day 3 and 5, with activation occurring on day 6. Macrophage-conditioned media were prepared by treating cells with IFNγ (12ng/mL; R&D Biosystems) for 24 hr, washing cells with PBS, and incubating them in serum-free media for an additional 24 hr. Protein lysate was collected in 1% SDS and quantified using a Pierce BCA protein assay kit (Thermo Scientific). For transcription inhibition experiments, cells were treated either with actinomycin D (2uM; Sigma) alone or in combination with IFNγ at 12ng/ml in serum-free RPMI for various time intervals up to 24h, following which media and cell lysate were collected. MSRN proteins in the macrophage-conditioned media was measured and normalized to total cellular protein, which were both quantified by immunoblotting.

Peritoneal macrophage isolation and activation – Peritoneal macrophages were harvested from the mice 5 days after 4% thioglycolate was injected. Cells were washed

with phosphate-buffered saline (PBS), seeded into 24-well plates (0.5×10^6/well), incubated at 37°C for 2 h in serum-free Dulbecco's minimum essential medium (DMEM), and washed 3 times with PBS. Macrophages were then cultured for 24h in DMEM, following which they were subjected to different treatments. Cells from diet-fed mice were either treated or harvest after 2h of plating post-isolation.

Macrophage siRNA treatment – Bone marrow-derived macrophages were transfected with control or *Stat1* siRNA (20 nM, Ambion) using Lipofectamine RNAiMAX reagent (Invitrogen) and analyzed 48h post-transfection as previously described (Becker et al., 2010). STAT1 knockdown was confirmed by immunoblot analysis.

Macrophage bacterial killing – Bone marrow-derived macrophages were incubated with *P. aeruginosa* for 2h to allow for phagocytosis, treated with gentamicin to kill non-internalized bacteria, lysed, and live bacteria were plated on agar to quantify the number of colony forming units (Hilbi et al., 2001).

Immunoblot analysis: Macrophage-conditioned medium was subjected to SDS-PAGE on 4%–20% gradient gels, transferred to PVDF membranes, and probed antibodies (0.5 µg/mL). Proteins were quantified by densitometry, using ImageJ software. Antibodies used for immunoblotting**:** rabbit anti-APOE (Abcam), goat anti-MFGE8 (R&D Systems), and rabbit anti-STAT1, rabbit anti-phospho-STAT1 (701) and rabbit anti-GAPDH (Cell Signaling).

qRT-PCR: RNA was isolated using QIAGEN Midi-Prep Kits and RT with Quantiscript (QIAGEN) using random hexamers (Invitrogen). mRNA levels were measured with specific primers (see Table 2.1) using SYBR green on a One Step Plus system (Applied Biosystems). Relative levels of each target gene were calculated using the ΔΔCt formula and 18S RNA as a control.

Statistics: For proteomics studies, differentially expressed proteins were identified using a combination of the unpaired two-tailed Student's *t*-test ($p < 0.05$) and *G*-test ($G > 1.5$) with correction for false discovery (FDR < 5%) as previously described (Heinecke et al., 2010). For all other studies, statistical significance ($p < 0.05$) was determined by an unpaired two-tailed Student's *t*-test using Prism GraphPad software (v.6.0h). Data are presented as means ± SEM.

Results

Obesity/IR-induced IFNγ targets the MSRN without inducing host defense genes.

We have shown that ablating IFNγ signaling in obese/IR mice resulted in correction of MSRN dysregulation and attenuation of macrophage cholesterol accumulation *in vivo* (see Figure 2.13A-C, Chapter 2). However, since the metabolic phenotype of *Ifngr1-/-* mice was unaltered (see Figure 2.13A, Chapter 2), these results suggest that IFNγ is required for obesity/IR to target MSRN proteins, which is consistent with the ability of this cytokine to target the network *in vitro*.

In addition to targeting the MSRN, IFNγ also alters many other genes/protein, many of which are critical to host defense (Dalton et al., 1993; Shtrichman and Samuel, 2001). To determine if those genes (i.e. *Irf1*, *Irf8*, *Ibp1*) were also regulated *in vivo*, we quantified their mRNA levels in elicited peritoneal macrophages from lean and obese/IR *wt* C57BL/6 and *Ifngr1-/-* mice. To our surprise, we found that none of these genes were induced by obesity/IR in *wt* C57BL/6 mice, nor were they lowered in macrophages from *Ifngr1-/-* mice (**Figure 3.1A**)**.** Moreover, phosphorylation of STAT1, a key signal transduction event in the IFNγ canonical pathway, was not observed in obese/IR *wt* mice (**Figure 3.1B**). Similarly, IFNγ target genes and phosphorylation of STAT1 was not induced in obese/IR, WTD-fed *Ldlr-/-* mice transplanted with *wt* bone marrow (**Figure 3.1C-D)** (see Figure 2.14, Chapter 2). Further, myeloid deletion of *Ifngr1* in these BMT mice did not result in changes to levels of IFNγ-target genes (**Figure 3.1E).** Together, these results suggest that IFNγ's effects on the MSRN in obese/IR mice occur in the absence of the induction of canonical IFNγ signaling.

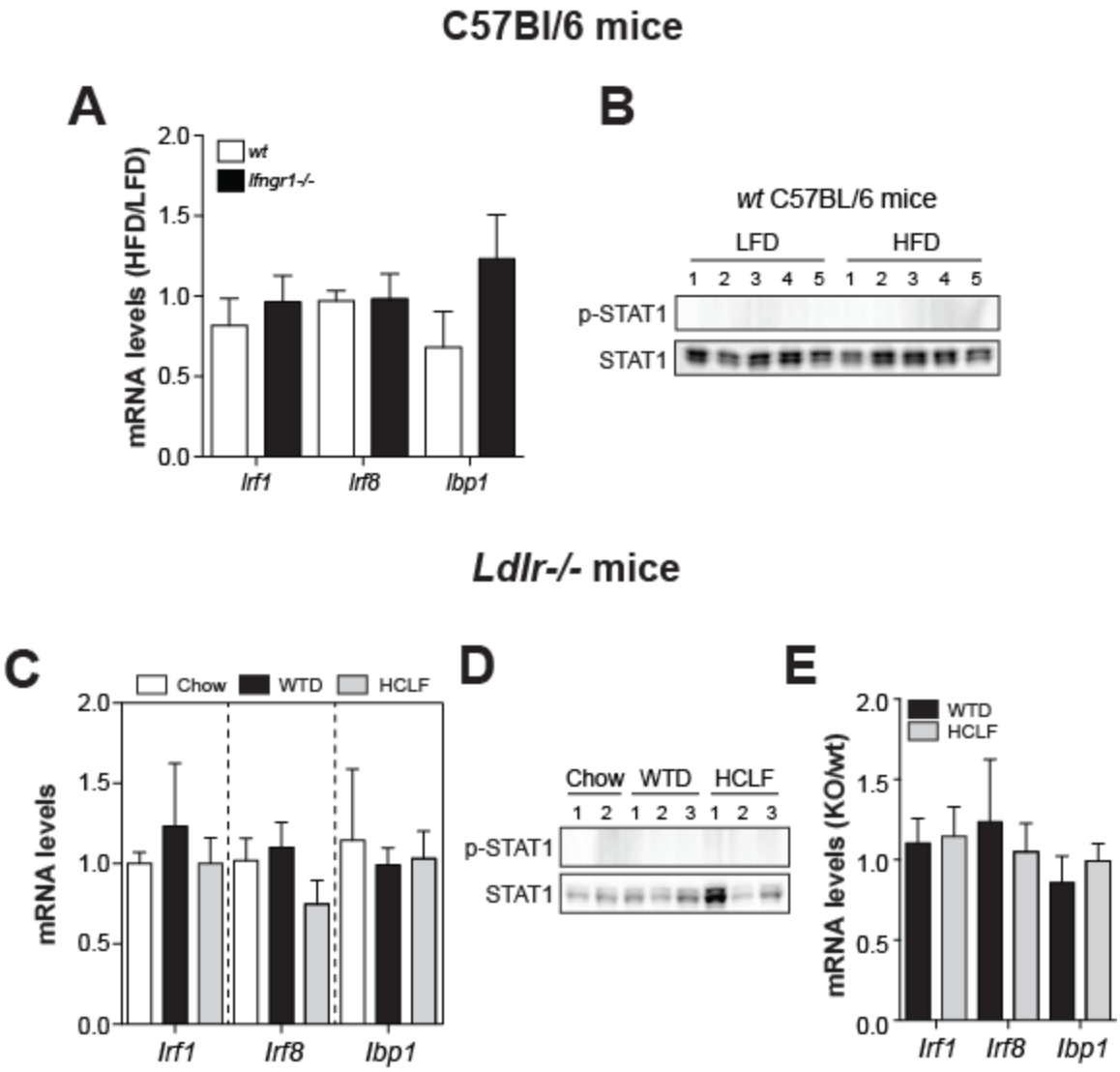


Figure 3.1: Ablating *Ifngr1* corrects MSRN proteins but does not correct canonical IFNγ-induce genes in obese/IR C57Bl/6 and *Ldlr-/-* BMT mice. *Panels A-B*: Peritoneal macrophages from w*t* and *Ifngr1-/-* mice were fed a LFD or HFD for 9 weeks. *Panel A:* IFNγ-target gene levels; *Panel B:* p-STAT1 and STAT1 levels. *Panels C-E*: Peritoneal macrophages from *Ldlr-/-* transplanted with w*t* and *Ifngr1-/-* bone marrow, were fed WTD or HCLF diets for 12-15 weeks. *Panel C:* IFNγ-target gene levels in *wt* mice; *Panel B:* p-STAT1 and STAT1 levels in *wt* mice; *Panel D:* IFNγ-target gene levels in *wt* and *Ifngr1-/-* mice. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3-12/group.

'Metabolic disease-appropriate' doses of IFNγ target MSRN proteins without inducing canonical IFNγ pathways.

Much of our understanding of how IFNγ affects macrophages is based on doses (~12 ng/ml) several orders of magnitude higher than those in patients with type 2 diabetes (~50 pg/mL) (Mirhafez et al., 2015; Nosratabadi et al., 2009), or those produced by T cells in obese/IR mice (see Figure. 2.10A, Chapter 2). We reasoned that doses of IFNγ found in obese/IR mice are sufficient to target MSRN proteins, but insufficient to induce host response genes and bacterial killing responses. Consistent with this hypothesis, low doses of IFNγ (30-120 pg/ml) suppressed APOE but neither induced STAT1 phosphorylation nor *Irf8* expression, all of which occurred at higher doses of IFNγ (**Figure 3.2A-C**). Moreover, macrophage functional responses such as killing of intracellular bacteria, *Pseudomonas aeruginosa*, was compromised at low doses of IFNγ (**Figure 3.2D**).

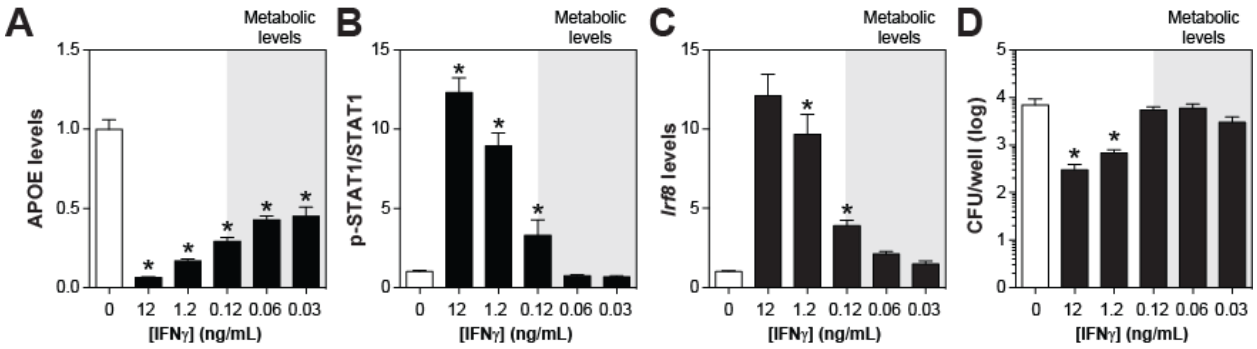


Figure 3.2: Low doses of IFNγ target the MSRN, without affecting host responses. *Panels A-D*: Macrophages were treated with increasing levels of IFNγ. *Panel A*: APOE levels. *Panel B*: p- STAT1/STAT1 levels. *Panel C*: *Irf8* levels. *Panel D*: Number of *P. Aeruginosa* killed by macrophages. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3-10/group.

IFNγ effects on MSRN and host defense are regulated by distinct pathways.

We know that the effect of IFNγ on the MSRN and host defense functions of macrophages are dependent upon interaction with IFNGR1. IFNγ associates with the ligand-binding IFNGR1 that heterodimerizes with IFNGR2, resulting in phosphorylation and nuclear translocation of STAT1 to induce interferon regulatory factors (IRFs) that up-regulate additional genes essential to host defense (Ikushima et al., 2013). Our demonstration that MSRN dysregulation and host defense pathways can be dissociated by altering IFNγ dose implies that distinct pathways (downstream of IFNGR1) control these diverse functional properties.

To test this hypothesis, we investigated whether knocking down *Stat1* with siRNA, or knocking out *Irf1* (*Irf1-/-*), two key components of the host defense pathway, could block IFNγ's effect on the MSRN. We treated siRNA-control, siRNA-*Stat1* (~80% knockdown, **Figure 3.3A**) or *wt* and *Irf1-/-* macrophages with 12 ng/mL IFNγ, as this high dose should be sufficient to ascertain whether STAT1 and/or IRF1 are required for IFNγ-induced MSRN dysregulation *in vitro*. We found that STAT1 and IRF1 were not required for APOE suppression, but required for IFNγ to induce bacterial killing (**Figure 3.3B-C**). These findings suggest that IFNγ targets MSRN proteins independent of canonical IFNγ signaling required for host defense, which is consistent with our *in vivo* findings (see Figure 3.1).

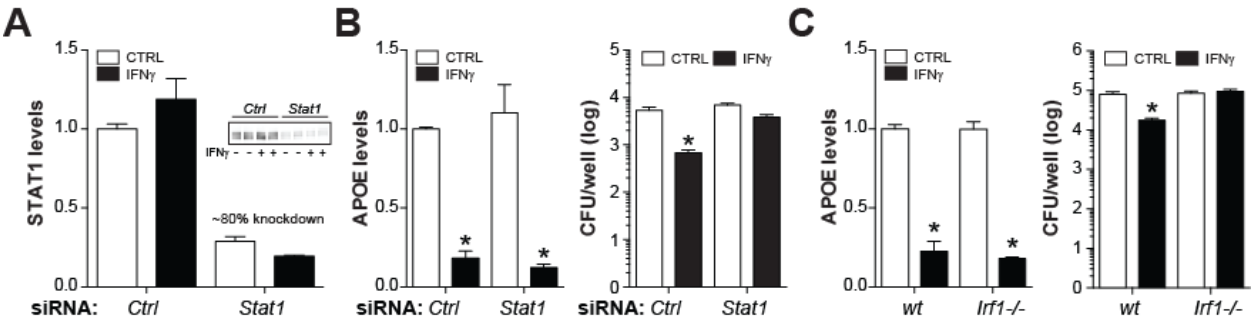


Figure 3.3: Canonical signaling is not required for IFNγ to alter MSRN proteins. *Panel A*: Efficiency of STAT1 knockdown in macrophages treated with control or *Stat1* siRNA. STAT1 levels were quantified 30min after IFNγ exposure. *Panels B-C*: Effects of IFNγ on APOE levels and number of *P. Aeruginosa* killed by siRNA control or siRNA *Stat1*macrophages (*Panel F*), and *wt* or *Irf1-/-* macrophages (*Panel G*). Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3-10/group.

IFNγ-induced MSRN dysfunction is transcription-dependent *in vitro*.

Our findings suggest that IFNγ-induced changes to the MSRN through a mechanism that is distinct from its canonical signaling pathway in macrophages. To explore mechanism by which IFNγ targets the MSRN, we first asked whether IFNγ could directly alter mRNA levels of the MSRN proteins. IFNγ did not alter mRNA levels of the MSRN proteins *in vitro* (**Figure 3.4A**), at high IFNγ doses (12 ng/mL). Moreover, mRNA levels for MSRN proteins were not altered in peritoneal macrophages from obese/IR mice (**Figure 3.4B**). These results suggest that IFNγ regulates MSRN proteins via a post-transcriptional mechanism.

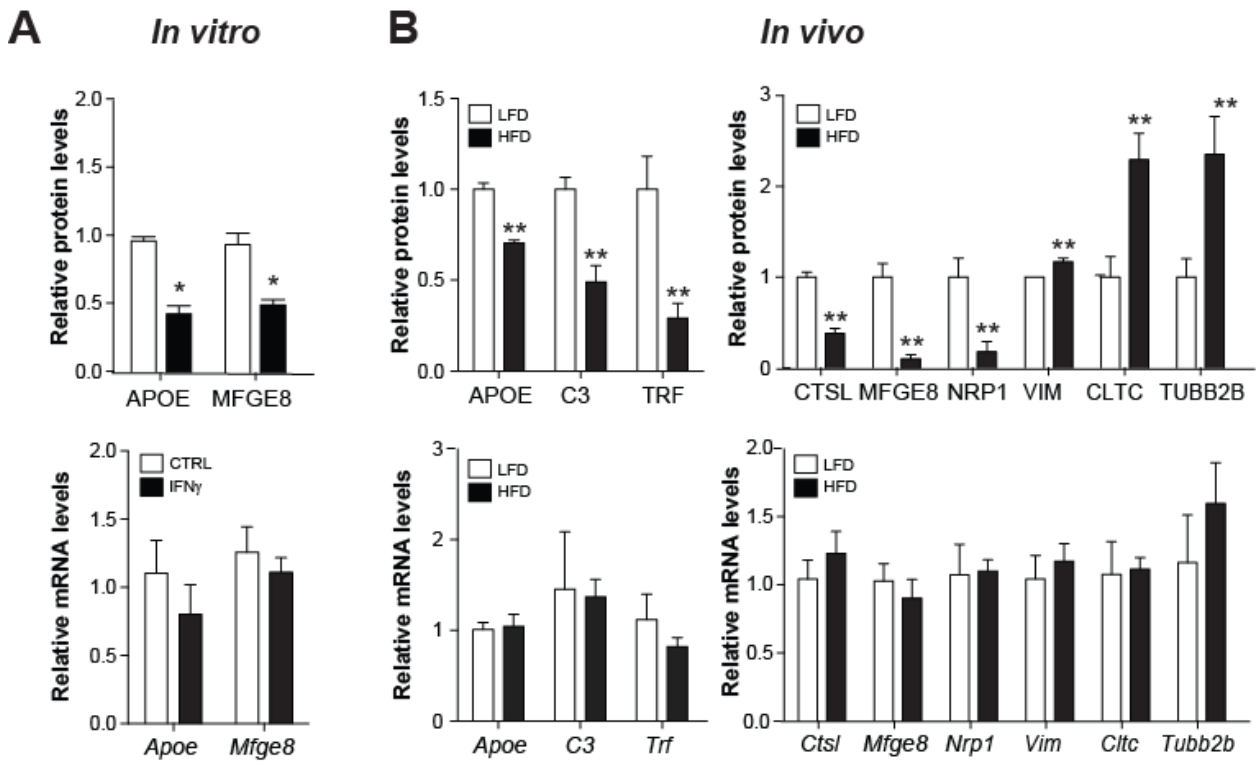


Figure 3.4: IFNγ does not alter mRNA levels of MSRN proteins *in vitro* and *in vivo*. Relative protein and mRNA levels of MSRN proteins from bone marrow-derived macrophages treated with IFNγ (*Panel A*) and peritoneal macrophages from lean and obese/IR *wt* mice (*Panel B*). Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3-5/group.

Next, to determine whether IFNγ-induced transcription was required for MSRN dysregulation, we treated macrophages with actinomycin D (Act D), a transcription inhibitor (Bensaude, 2011), to block new transcription of proteins post-treatment. For this, macrophages were pre-treated with either with IFNγ (12ng/ml), Act D (2uM) alone or Act D + IFNγ, following which conditioned media was collected over a duration of 24h at regular intervals. Analysis of macrophage secreted media revealed that IFNγ-induced APOE suppression occurred as early as 8h and was persistent up to 24h post-treatment (**Figure 3.5**). However, in the presence of Act D, this IFNγ effect on the media APOE

was abolished, suggesting that transcription is required for IFNγ induces a protein ('candidate protein(s)') as early as 8h post exposure that post-transcriptionally targets the MSRN.

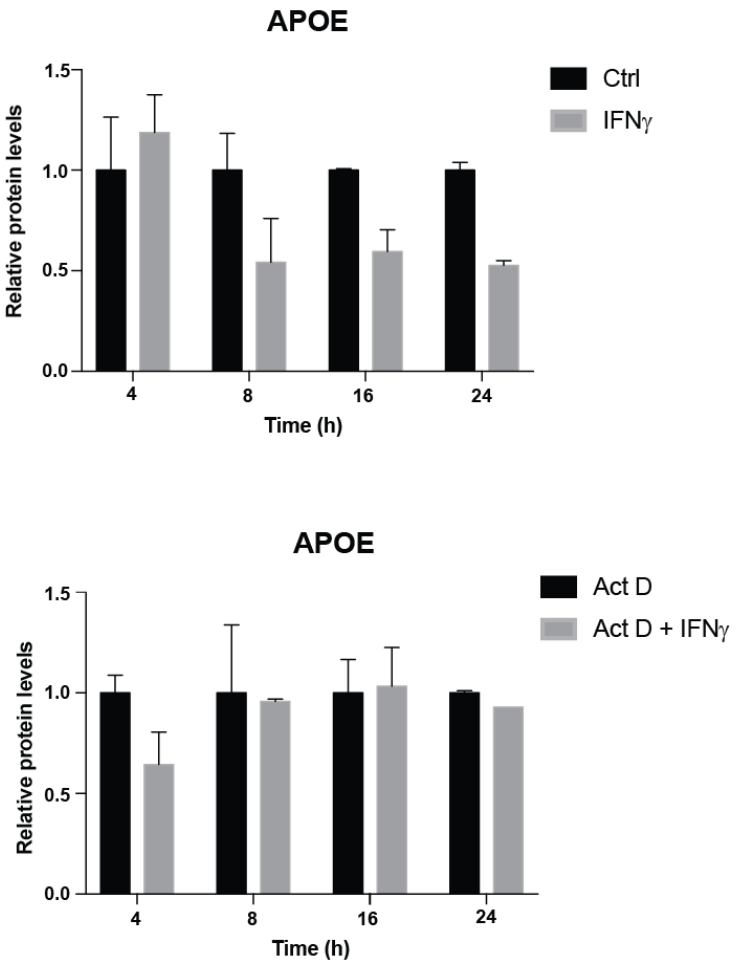


Figure 3.5: IFNγ targets MSRN proteins by a post-transcriptional manner. Bone marrow-derived macrophages treated with control, IFNγ, (*top panel*) Act D (2uM) or ActD + IFNγ. (*bottom panel*). Media and cell lysate were collected and analyzed for APOE and GAPDH levels, respectively. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=2/group.

Discussion

Although IFNγ plays an important role in atherosclerosis (Boshuizen and de Winther, 2015; Voloshyna et al., 2014; Yu et al., 2015), it is a poor therapeutic target because of its pivotal role in host defense (Dalton et al., 1993; Shtrichman and Samuel, 2001). Canonical signaling, wherein IFNγ binds to IFNGR1, causing heterodimerization with IFNGR2 and subsequent phosphorylation and translocation of STAT1 into the nucleus resulting in regulating of target genes such as IRF1, is essential to drive host defense immune responses (Ikushima et al., 2013). Surprisingly, none of these genes (e.g. *Irf1*, *Irf8*, *Ibp1*) were induced nor was STAT1 phosphorylation observed in macrophages from obese/IR mice even though IFNγ-induced MSRN dysregulation occurred. Moreover, together with the observation that none of these IFNγ-target genes were altered in obese/IR mice that lacked *Ifngr1*, our findings suggest that the pro-atherogenic actions of IFNγ on macrophages are independent of its canonical signaling pathway.

Previous studies have showed that IFNγ alters transcription of many genes in STAT1-/- macrophages (Gil et al., 2001), suggesting that STAT1-independent mechanisms significantly contribute to IFNγ's effects on macrophage gene expression and function. However, the impact of these transcriptional changes on macrophage function and diseases are largely unknown. Our findings show that the STAT1-IRF1 component of canonical signaling is not required for IFNγ to cause MSRN protein dysregulation and increased cholesterol accumulation in macrophages, two functions that are mechanistically linked to atherosclerosis. This is supported by *in vivo* studies wherein mice specifically lacking either *Stat1* or *Ifngr2*, two key components of

canonical IFNγ signaling pathway, failed to attenuate atherosclerosis (Boshuizen et al., 2016; Lim et al., 2008). Taken together, this suggests that IFNγ's role on atherosclerosis progression and its effects in regulating host defense responses occur through mechanistically distinct pathways.

IFNGR1 is essential for IFNγ signaling and we confirmed that IFNγ cannot target MSRN proteins in *Ifngr1-/-* macrophages *in vitro* (see Figure. 2.8A, Chapter 2). How could blocking IFNγ signaling *in vivo* normalize MSRN proteins without altering canonical IFNγ-induced genes? Much of our understanding of how IFNγ affects macrophages is based on doses (~12 ng/ml) several orders of magnitude higher than those in patients with type 2 diabetes (~50 pg/mL) (Mirhafez et al., 2015; Nosratabadi et al., 2009). This is also observed in vivo wherein IFNγ levels produced by activated splenic T cells from obese/IR mice were comparable to those circulating levels of IFNγ in type 2 diabetic patients (see Figure. 2.10A, Chapter 2). We reasoned that doses of IFNγ found in obese/IR mice are sufficient to target MSRN proteins, but insufficient to induce host response genes and function. Two lines of evidence support this hypothesis. First, plasma levels of IFNγ in wild-type or *Ldlr-/-* mice fed various diets fell below detection limit of the assay (<100pg/ml). Second, although 'infectious doses' of IFNγ induced STAT1 phosphorylation, *P. aeruginosa* killing and targeted MSRN proteins (e.g. APOE), 'metabolic disease-appropriate' doses of IFNγ could still target MSRN proteins, but could not support STAT1 phosphorylation or increased bacterial killing.

Our work identifies a novel, host defense-independent pathway through which IFNγ asserts its pro-atherogenic actions on macrophages. Future work aimed at identifying the protein(s) that post-transcriptionally alter MSRN proteins and increases

macrophage cholesterol accumulation is important to develop new therapeutics that target pro-atherogenic properties of IFNγ biology without interfering with host defense.

CHAPTER FOUR: DISCUSSION AND FUTURE DIRECTIONS

Discussion

T2D results in an 2-4 fold increased risk of atherosclerosis, with a mortality rate of nearly 70% from CVDs. Despite these statistics, the underlying mechanisms for the increased risk in type 2 diabetic patients is poorly understood. Type 2 diabetic patients have increased plasma cholesterol levels and the increased atherosclerotic risk in these patients is conferred independent of plasma cholesterol levels (Costa et al., 2006). In mice, feeding atherosclerosis-prone *Ldlr-/-* mice diabetogenic diets also results in increased plasma cholesterol levels (Hartvigsen et al., 2007). Thus, the major challenge with studying T2D-associated atherosclerosis has been in deciphering the direct effects of obesity/IR on atherosclerosis progression in the presence of hypercholesterolemia.

In order to address this challenge, we used a combination of genetic and dietary strategies in this study. Using a proteomics approach, we have shown that obesity/IR targets a pro-atherogenic macrophage protein network, called the macrophage sterol-responsive network (MSRN), to promote increased macrophage cholesterol accumulation and atherogenesis, in the presence of hypercholesterolemia. We also found that the obesity/IR-induced macrophage effects was due to IFNγ as ablating IFNγ signaling (*Ifngr1-/-*) in obese/IR corrected the MSRN protein dysregulation and increased cholesterol accumulation observed in macrophages isolated from *wt* obese/IR mice. Moreover, the observed correction in obese *Ifngr1-/-* mice occurred without affecting the metabolic parameters in comparison to *wt* obese/IR mice. This is important because our data shows that obesity/IR primes T cells to produce IFNγ and so if deletion of *Ifngr1-/-* resulted in any changes to the extent of obesity/IR, this approach to

measure the direct impact of obesity/IR-induced IFNγ on macrophages in obese/IR *Ifngr1-/-* mice would not be feasible. Additionally, the decrease in macrophage cholesterol levels could not be explained by altered lipid metabolism as obese/IR *Ifngr1-/-* mice did not exhibit any changes in these genes.

Transplanting atherosclerotic *Ldlr-/-* mice with bone marrow from either *wt* or *Ifngr1-/-* mice allowed us to assess the physiological importance of macrophage IFNγ signaling on atherosclerosis progression in the context of hypercholesterolemia (HC) only, and in the presence of both obesity/IR and HC. Myeloid-specific deletion of *Ifngr1* protected against MSRN dysregulation and increased macrophage cholesterol accumulation only when obesity/IR was present but didn't have any affect in the presence of hypercholesterolemia alone. Also, aortic root lesion area was lower in *Ifngr1-/-* bone marrow-transplanted (BMT) animals compared to *wt*, only when obesity/IR was present. Moreover, lesion area from WTD-fed *wt* BMT mice, that are both obese/IR and hypercholesterolemic, positively correlated with plasma insulin levels and not plasma cholesterol levels. Importantly, this relationship was abolished in WTD-fed *Ifngr1-/-* BMT mice, suggesting that myeloid IFNγ signaling is required for obesity/IR to impact artery wall macrophages. Thus, our findings indicate that obesity/IR-induced IFNγ is the main driver of MSRN dysfunction to promote increased macrophage foam cell formation and exacerbates atherosclerosis.

Although our data shows that obesity/IR results in an increased production of IFNγ by activated splenic T cells, one of the major limitations of this work is the identification of a definitive source of IFNγ in the artery wall in the different models used in this study. IFNγ is one of the main pro-inflammatory cytokines in the lesion. It affects

different cell types in the artery wall and regulates various functions in these cells. It has been shown to be secreted by various cells like, dendritic cells (DCs), natural killer (NK) cells, T cells, NKT cells, macrophages and smooth muscle cells (SMCs), that are present within the lesion and contribute to the pathogenesis of atherosclerosis (Getz and Reardon, 2017; Tenger et al., 2005). The importance of IFNγ in atherosclerosis is highlighted by studies in *Ldlr-/-* mice wherein deletion of *Ifng* or *Ifngr* resulted in decreased lesion development whereas exogenous IFNγ administration enhanced atherosclerosis (Buono et al., 2003; Gupta et al., 1997; Whitman et al., 2000).

Many studies have shown that both in human and murine atheromas, T-cells, particularly a distinct subset of pro-inflammatory CD4+ helper T (Th1) cells, are the major producers of IFNγ (Frostegard et al., 1999; Stemme et al., 1995). Deletion of T-bet, a transcription factor necessary for the differentiation of Th1 subset of T cells, has been shown to reduced atherosclerosis development in *Ldlr-/-* mice (Buono et al., 2005). Using *Rag1-/- Ldlr-/-* mice, which lack both T cells and B cells, the absence of both adaptive immune cells resulted in a significant reduction in early atherosclerotic lesion development (Lichtman, 2013). Studies in *Apoe-/-* mice have shown that up to 40% of the CD4+ T cell population in lesions produce IFNγ (Li et al., 2016) and a decrease in lesion T cell numbers by treating with chemokine receptor-antagonist,TAK-779, resulted in a concurrent decrease of 98% in IFNγ levels within the lesion (van Wanrooij et al., 2005).

Although IFNγ is the main cytokine involved in atherosclerosis progression, other pro-inflammatory cytokines such as IL-12, IL-18, TNF-α, also contribute to lesion development under hypercholesterolemic conditions (Ramji and Davies, 2015; Witztum

and Lichtman, 2014). The contribution of these their pro-inflammatory cytokines may explain the development of lesions observed in *Ifngr1-/- Ldlr-/-* BMT mice fed the HCLF diet (see Figure 2.16F, Chapter 2). Like macrophages, T cells are highly plastic and can be reprogrammed by extracellular cues in the environment to aide in adaptive immune responses (Bluestone et al., 2009). T cell plasticity can be observed in atherosclerotic lesions wherein heterogeneous T cell populations produce different cytokines that attribute both pro- and anti-inflammatory functions in disease pathogenesis. Other helper T cell subsets such as Th2 and T17 and regulatory T cell (Tregs) have also been shown to influence atherosclerotic lesion development (Tabas and Lichtman, 2017; Witztum and Lichtman, 2014).

A specialized subset of CD4+ T-cells called, natural killer T (NKT) cells, first described by Bendelac and colleagues in 1994 (Bendelac et al., 1994), have been increasingly studied for their role of atherosclerosis in the last decade. These cells represent a link between innate and adaptive immune systems and are so called as they express cell-surface markers characteristic of both NK cells as well as those of T cells (Getz and Reardon, 2017). Of especial relevance to atherosclerosis is that these NKT cells respond to both exogenous and endogenous lipid antigens such as glycolipids and phospholipids, presented by non-classical major histocompatibility complex (MHC) class I molecules of the CD1 family on antigen-presenting cells (APCs) (Getz and Reardon, 2017). Activation of classical NKT cells or iNKT cells, results in the rapid production of Th1 cytokines, such as IFNγ, TNFα and Th2 cytokines such as IL4, that have been shown to influence atherosclerotic lesion development in several ways (Getz and Reardon, 2017; Tupin et al., 2007; Van Kaer et al., 2011). WTD-fed *Ldlr-/-*

mice lacking NKT cells (*Jα18-/- Lldr-/-*) have been shown to have decreased lesion area in the aortic root as well as ascending aorta (Rogers et al., 2008). Conversely, overexpression of NKT cells such as in high-fat fed Vα14 transgenic *Ldlr-/-* mice, resulted in a two-fold increase in atherosclerosis lesion formation in the aorta along with increased dyslipidemia and insulin resistance compared to *Ldlr-/-* mice (Subramanian et al., 2013). Thus, since T cells are abundant in the adventitia and perivascular tissue (Majesky, 2015; Omar et al., 2014), and can be activated by glycosylated lipid ligands, evaluating iNKT cells in perivascular adipose tissue may provide insight into their role in the pathogenesis of atherosclerosis, especially in the context of T2D.

Murine studies of diet-induced obesity/IR (DIO) have shown that T cells infiltrate visceral adipose tissue and promote pro-inflammatory phenotypes in macrophages and contribute to systemic IR (McLaughlin et al., 2014). In humans, the pro-inflammatory milieu in obese adipose tissue has been shown to promote foam cell formation in adipose tissue macrophages (Shapiro et al., 2013). Thus, though both T cells and macrophage phenotypes are driven by their environmental cues, concerted functioning of these two cells in adipose tissue to promote inflammation and enhance macrophage foam cell formation suggests that similar interactions may occur in atherosclerotic lesions. Since isolation of T cells from atherosclerotic lesions is challenging, analysis of T cell subsets in obese adipose tissue of WTD-fed *Ldlr-/-* mice could provide insight into specific T cell subsets and mechanisms, that could promote inflammation within the atheroma.

Another limitation of our work is that the analysis of obesity/IR-induced macrophage dysfunction *in vivo* was mainly in elicited peritoneal macrophages.

Although peritoneal macrophages have been shown to be reasonable surrogates for artery wall macrophages (Becker et al., 2010; Li et al., 2004), additional studies are required to shown that obesity/IR can produce similar dysfunction in artery wall macrophages, as macrophage phenotypes are largely driven by tissue-specific microenvironments (Gordon and Taylor, 2005). Our approach to isolate and quantify MSRN proteins from murine artery wall macrophages is promising, as we was able to quantify changes in levels of APOE and MFGE8 caused by exogenous injection of IFNγ in *Ldlr-/-* mice. However, additional work to determine obesity/IR-induced MSRN protein dysregulation in artery wall macrophages from WTD-fed *Ldlr-/-* mice, is required.

Arterial lipid retention occurs mainly due to properties of hemodynamics, at sites of branch points or bifurcations of major vessels, where disturbed blood flow is inevitable (Frangos et al., 1999). In the work presented here, we have evaluated atherosclerotic lesion area only in the aortic root of bone marrow-transplanted *Ldlr-/-* mice. This was due to the absence of lesions observed in other sites such as in the innominate artery, ascending and descending aorta. The absence of lesions at these sites could be due to the damaging effects of irradiation used to deplete the endogenous bone marrow prior to transplantation. In order to circumvent this issue of irradiation, I have successfully generated *Ldlr-/-* mice with macrophage-specific deletion of *Ifngr1* (*Ldlr-/- Ifngr1 fl/fl LysMcre+/-*). Assessment of atherosclerosis in control (*Ldlr-/- Ifngr1 fl/fl LysMcre+/+) and* macrophage-specific *Ifngr1-/- (Ldlr-/- Ifngr1 fl/fl LysMcre+/-)* mice fed WTD and HCLF diets will help us determine the impact of obesity/IR-induced IFNγ on atherosclerosis in other arterial sites as well. This is important because, mice and humans do not develop atherosclerosis at same arterial

sites (Getz and Reardon, 2012; VanderLaan et al., 2004). Humans develop lesions in many arteries including the coronary artery whereas the primary sites for atherosclerosis in mice has mostly been studied in aortic root and innominate artery (VanderLaan et al., 2004). Also, since one of the future directions of this work is to determine the impact of T2D in human artery wall macrophages (detailed below), it will be essential to evaluate lesion in other sites in mice so that we can compare T2D-induced MSRN dysregulation in murine and human artery wall macrophages, at similar although not identical sites.

Although the work described here is focused on understanding the mechanisms of T2D-associated atherosclerosis in obesity/IR, our findings can be extended to other types of diabetes mellitus and autoimmune diseases as well. Non-obese type 2 diabetic patients have also been reported to have increased levels of pro-inflammatory cytokines compared to healthy controls (Goyal et al., 2012) and they have a similar increased risk of cardiovascular disease as obese T2D patients (Vaag and Lund, 2007). Patients with autoimmune, inflammatory diseases such as type1 diabetes mellitus (T1DM), systemic lupus erythematosus and rheumatoid arthritis have also been reported to have high risk for atherosclerosis (de Ferranti et al., 2014; Frostegard, 2005; Sherer and Shoenfeld, 2006). Macrophage MSRN dysregulation and increased foam cell formation due to elevated levels of pro-inflammatory cytokines such as IFNγ, could explain the increased atherosclerotic risk observed in the aforementioned pathologies, thereby highlighting the broad implications of our study.

The impact of IFNγ signaling in all different cell types involved in atherosclerosis such as macrophages, endothelial cells, smooth muscle cells, etc., have been extensive

studied in the progression of this disease. For instance, similar to our findings, IFNγ has been shown to cause a reduction in APOE levels without changes to mRNA levels in human THP-1 cells, a monocyte cell line that can differentiate into macrophages, and deletion of IFNγ in myeloid cells has resulted in decreased atherosclerotic lesion size in *Apoe*-deficient mice (Brand et al., 1993; Gupta et al., 1997) . Additionally, IFNγ has been demonstrated to play a role in macrophage lipid metabolism as well. Incubation of macrophages with IFNγ has been shown to cause downregulation of ABCG1 transport protein and impede in reverse cholesterol transport to promote foam cell formation in human THP-1 cells (Panousis and Zuckerman, 2000; Reiss et al., 2004). However, the underlying mechanisms delineating the IFNγ signal transduction events that regulate these crucial macrophage functions to promote atherosclerosis are incompletely understood.

Canonical IFNγ signaling is initiated by ligand binding to IFNGR1 causing its heterodimerization with IFNGR2, leading to a cascade of phosphorylation events that allow for signal propagation within the cell (Ikushima et al., 2013). Phosphorylation of receptor-bound STAT1 results in its dimerization and translocation into the nucleus for transcription of IFNγ-target genes (Ikushima et al., 2013). Interestingly, obesity/IR did not trigger expression of IFNγ-target genes and did not result in phosphorylation of STAT1, even though MSRN proteins were altered. One possible explanation could be the dose of IFNγ was insufficient *in vivo*. In type 2 diabetic patients, IFNγ levels are much lower (60-120pg/ml) (Mirhafez et al., 2015; Nosratabadi et al., 2009) than is typically used in *in vitro* studies which reflects doses used for studying innate immunity in infection models (12ng/ml). Accordingly, our data shows that ‘metabolically-

appropriate' doses of IFNγ can target MSRN proteins but are insufficient to produce crucial, functional IFNγ responses required for host defense. How do low and high doses of IFNγ regulate different signaling events within macrophages? One possible explanation is that the lower concentration of IFNγ, influences differential receptor interactions such as IFNGR1-IFNGR1 as opposed to IFNGR1-IFNGR2 as occurs in the case of high concentrations of IFNγ. The differential homodimerizations of the receptor might result in the activation of non-canonical IFNγ signal transducers. Future studies aimed at evaluating the binding affinity of IFNγ to its receptors at different doses will enable the understanding the specificity of 'metabolic-appropriate' doses of IFNγ to cause MSRN dysregulation but not induce host defense responses.

In our studies, IFNγ altered MSRN proteins even in the absence of IRF1 or at diminished levels of STAT1, two key transcription factors in canonical IFNγ signaling (Majoros et al., 2017). This suggests that dysregulations of these proteins occurs through a non-canonical, host defense-independent signaling pathway. Although our *in vitro* results using STAT1 siRNA have to be validated in STAT1 KO macrophages, our data supports previous *in vivo* findings from other groups, where myeloid-specific deletion of STAT1 in *Ldlr-/-* mice did not protect against atherosclerosis (Lim et al., 2008). Similarly, macrophage deficiency of IFNGR2, the non-ligand binding receptor that is required for signal transduction, also did not significantly decrease atherosclerotic lesion area in *Ldlr-/-* mice (Boshuizen et al., 2016). However, our findings show that myeloid deficiency of IFNGR1 resulted in a reduction in atherosclerotic lesion area in WTD-fed *Ldlr-/-* mice. We have also shown that IFNGR1 is required for IFNγ-induced suppression of APOE *in vitro*. Furthermore, since mRNA levels of MSRN protein were

unchanged both *in vitro* and *in vivo*, together, our findings suggest that a post-transcriptional regulatory mechanism is operative in this host defense-independent pathway.

Thus, the work described in this dissertation highlights a novel IFNγ-driven mechanism through which obesity/IR impacts artery wall macrophages to promote atherogenesis. Identification of the key regulator(s) through which IFNγ targets MSRN proteins to promote increased cholesterol accumulation in MSRN-dysregulated macrophages is crucial to the understanding of the pathogenesis of atherosclerosis in obesity/IR. Further delineation of the mechanisms of this host defense-independent pathway through which IFNγ promotes pro-atherogenic functions in macrophages may lead to development of novel therapeutics to alleviate atherosclerotic risk without compromising immunity in type 2 diabetic patients.

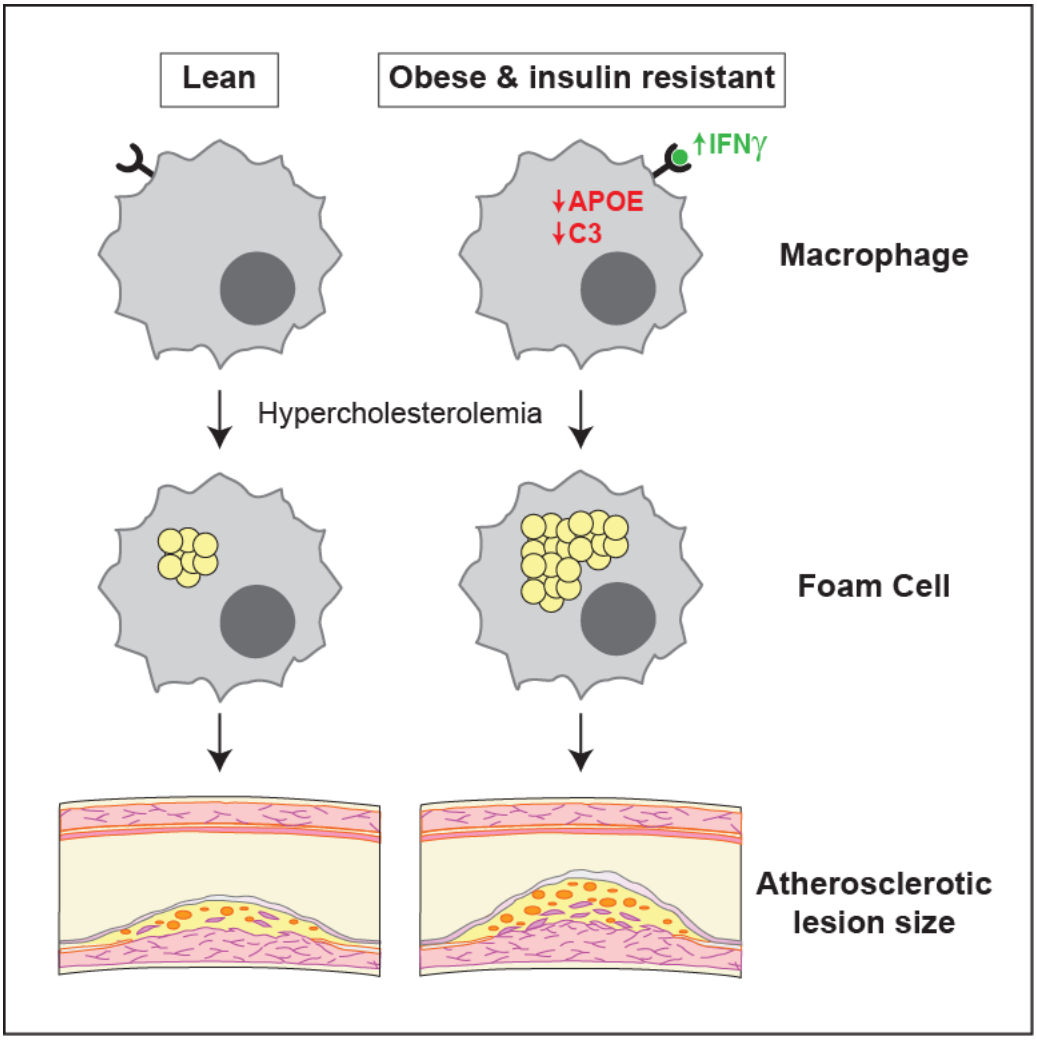


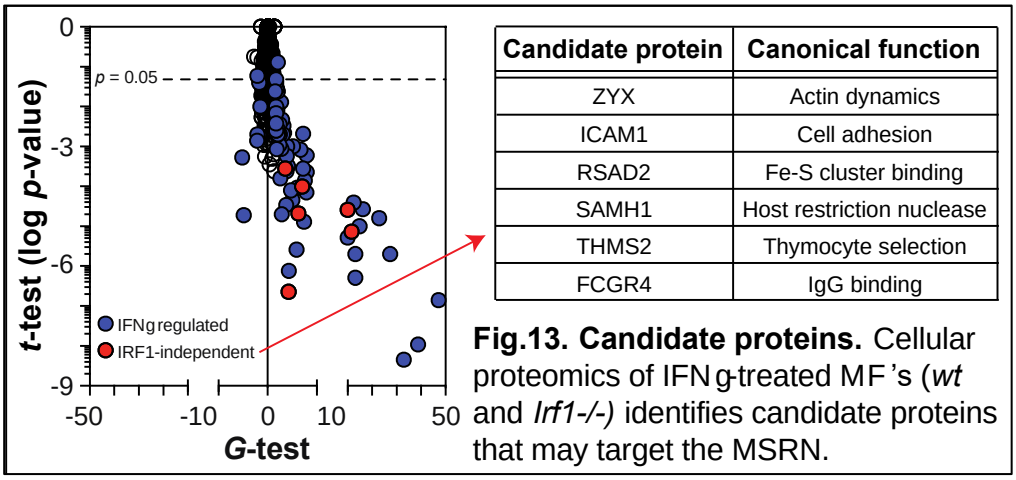
Figure 4.1: A model for how obesity and IR promote atherosclerosis.

Future Directions

The following sections outline future directions with preliminary data for (i) understanding the mechanisms through which IFNγ targets the MSRN in macrophages, and (ii) evaluating how T2D promotes atherosclerotic risk in patients by studying human artery wall macrophages using proteomics.

Delineating mechanism of host defense-independent, IFNγ-regulated pathway for MSRN dysfunction and increased cholesterol accumulation in macrophages.

To delineate the mechanism and identify the 'candidate protein' through which IFNγ alters the MSRN post-transcriptionally, we used a discovery-based proteomics approach. Since our data indicate that STAT1-IRF1 component of canonical IFNγ signaling is not required to target the MSRN, we analyzed whole cell proteomes from *wt* and *Irf1-/-* macrophages exposed to vehicle or IFNγ (12 ng/mL) for 6h and identified 4403 proteins. IFNγ significantly altered the levels of 77 proteins in *wt* macrophages, and this list was narrowed down to 6 candidate proteins by eliminating proteins that were normalized in *Irf1-/-* macrophages exposed to IFNγ (**Figure 4.2**).



Candidate protein	Canonical function
ZYX	Actin dynamics
ICAM1	Cell adhesion
RSAD2	Fe-S cluster binding
SAMH1	Host restriction nuclease
THMS2	Thymocyte selection
FCGR4	IgG binding

Fig.13. Candidate proteins. Cellular proteomics of IFNg-treated MF's (*wt* and *Irf1-/-)* identifies candidate proteins that may target the MSRN.

Figure 4.2: Cellular proteomics of IFNγ-treated *wt* and *Irf1-/-* macrophages identifies candidate proteins that may target the MSRN. *Left panel*: Blue circles indicate all proteins that are regulated by IFNγ, black and red circles indicate IFNγ-regulated proteins that are IFR1-dependent and -independent, respectively. *Right panel*: Identity and function of IRF1-independent candidate proteins (red circles in left panel).

We are in the process of evaluating whether these proteins are responsible for IFNγ-induced MSRN dysfunction *in vitro* using bone marrow-derived macrophages from knockout mice. One of the candidate proteins tested in this IRF1-independent pathway, was zyxin (ZYX), a zinc-binding protein that binds and regulates the actin cytoskeleton (Han et al., 2017; Smith et al., 2014). Previous studies showed that ZYX regulates actin-based vesicle rocketing in macrophages (Southwick et al., 2003), and in our previous study, we found that many MSRN proteins localized to extracellular vesicles (Becker et al., 2010), suggesting that MSRN proteins may be regulated by vesicle release.

Bone marrow-derived macrophages isolated from *wt* and *Zyx-/-* mice were treated with IFNγ and their secreted media was analyzed for APOE suppression by western blot. If zyxin is a key regulator of the MSRN via IFNγ, then IFNγ-induced APOE suppression should not be observed in *Zyx-/-* mice. However, our data showed that that zyxin was not involved in the alternate pathway as APOE was suppressed by IFNγ even in the absence of zyxin (**Figure 4.3A**). Similarly, another candidate, intracellular adhesion molecule-1 (ICAM1), was tested for IFNγ-induced APOE suppression using macrophages from *Icam1-/-* mice and determined not to be involved in this putative IFNγ-driven, IRF1-independent pathway (**Figure 4.3B**).

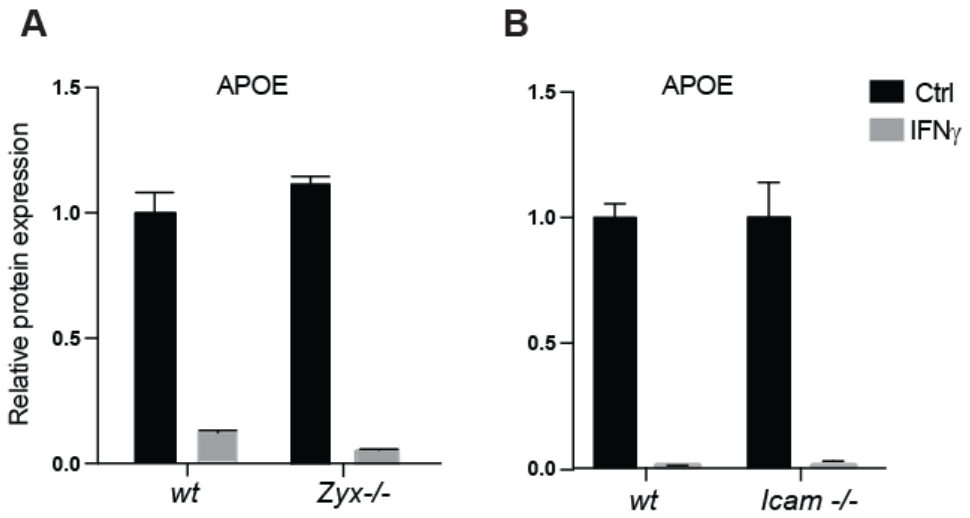


Figure 4.3: Analysis of potential candidates of IFNγ-induced IRF1-independent pathway. Bone marrow-derived macrophages from *wt*, *Zyx -/-* (Panel A) and *Icam-/-* (Panel B) mice were treated with 12ng/ml IFNγ. Conditioned media and protein lysate were collected and blotted for APOE and GADPH respectively. Results are mean ± SEM. *, $p<0.05$ (*t*-test); n=3/group.

We are in the process of acquiring knockout mice or generating siRNA targets for our remaining candidate proteins, following which they will be tested in a similar manner for their involvement in this IRF1-independent pathway for IFNγ-induced APOE suppression *in vitro*. If none of these six candidates are involved (since proteomics does not detect all proteins), we will perform RNA seq to identify additional potential candidates and test them using the same methodology as our current screening approach. Another possibility is to use phospho-proteomics to begin to identify the non-canonical signaling pathway through which IFNγ alters MSRN proteins. Candidate proteins are ones that will be phosphorylated within 30 minutes of IFNγ treatment in *wt* and *Stat1-/-* macrophages and will lead to the subsequent transcription of other protein(s) that cause APOE suppression within 8 hours of addition of IFNγ. Additionally,

candidates of this pathway should not be able to induce host defense immune responses in macrophages as, based on our findings, the pro-atherogenic and anti-bacterial effects of IFNγ function through distinct pathways in macrophages

Once identified, the importance of this 'candidate protein' in macrophages, in the context of atherosclerosis will be determined in the presence and absence of obesity/IR using bone marrow transplantation or macrophage specific deletion. Although this is a very exciting direction for the project, the timeline associated with this line of discovery is beyond the scope of my graduate school tenure. These studies will be continued by current members and new additions to the lab.

Determine the effect of type 2 diabetes on MSRN proteins in human artery wall macrophages.

Our ultimate objective is to determine if a macrophage IFNγ-MSRN pathway may help to explain how T2D promotes atherosclerosis in humans. Our work thus far has determined that macrophage IFNγ signaling is required for obesity/IR to promote atherogenesis in mice, through a host defense-independent pathway. The critical next step is to extend these findings to patients. We have already shown that in human monocyte-derived macrophages (HMDMs), IFNγ suppresses secretion of APOE, a key MSRN protein (see Figure 2.8, Chapter 2). Moreover, our established methodology to isolate, purify and evaluate murine artery wall macrophages (MAMs) (see Figure 2.10, Chapter 2) gives us a good start to utilize the tools we have developed to enable us for similar translational work from human patient samples. To this end, we have

successfully isolated human artery wall macrophages (HAMs) from patients undergoing carotid endarterectomy procedures and have interrogated them by proteomics. Thus far, in two patients, we have identified ~800 HAM proteins, including 8 of the 9 MSRN proteins, along with many others that are implicated in atherosclerosis (**Figure 4.4**).

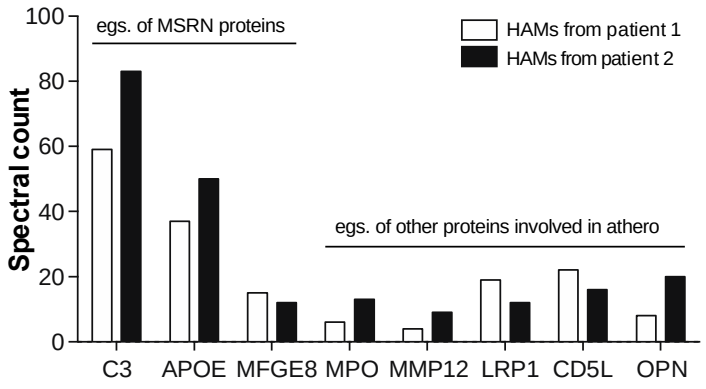


Figure 4.4: Proteomics analysis of HAMs quantifies proteins implicated in atherosclerosis. Spectral counts of MSRN and non-MSRN proteins known to be involved in atherosclerosis from human artery wall macrophages (HAMs) from two patients, represented by open or colored bars.

Analysis of MSRN dysfunction from a mixed cohort of diabetic and non-diabetic patients will enable us to compile an initial data set to begin exploring relationships between specific HAM protein(s) and the clinical presentation of atherosclerotic disease. This should provide a better understanding of macrophage dysfunction and atherosclerosis risk in diabetic patients. Along with measuring IFNγ levels in these patients, statistical correlation analyses would help us evaluate whether IFNγ regulates this pro-atherogenic network in humans as well, suggesting that MSRN dysregulation is common to both human and murine atherosclerotic disease. This data would support

our model wherein IFNγ induction in type 2 diabetics promotes MSRN protein dysregulation, which in turn drives macrophage cholesterol accumulation and atherogenesis.

It is possible that HAMs from type 2 diabetic patient do not exhibit MSRN dysregulation. We expect this to be unlikely since obesity/IR promotes MSRN dysregulation in mice and IFNγ similarly regulates certain MSRN proteins (e.g. APOE) in mouse and human macrophages. However, in the case of this outcome, we would use our unbiased proteomics approach to assess other functional processes known to influence atherosclerosis (e.g. inflammation, lipid metabolism) in type 2 diabetics relative to non-diabetics.

Understanding how T2D promotes macrophage dysfunction is critical to developing therapeutics to alleviate risk in this highly susceptible patient cohort. Irrespective of the nature of the outcome of these studies, because our knowledge of HAMs is limited, our proteomics characterization of HAMs obtained from patients of varying age, sex, plaque complexity, and blood measures (e.g. cytokines, lipids) will provide us, and the research community, with a rich data set to begin exploring relationships between HAM protein(s) and the clinical presentation of atherosclerotic disease.

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