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METABOLICALLY ACTIVATED ADIPOSE TISSUE MACROPHAGES AND INSULIN RESISTANCE

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Dedication

I would like to dedicate this work to my parents. This work was only possible because of their unending love and support.

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LIST OF ABBREVIATIONS

ABCA1- ATP binding cassette subfamily A member 1

Ad-CM – Adipose tissue conditioned media

AKT – protein kinase B

ATM – Adipose tissue macrophage

BMDM – Bone marrow derived macrophage

BMI - Body mass index

BTK – Bruton’s tyrosine kinase

CD36 – Fatty acid translocase

CLS- Crown-like structure

COX2- Cyclooxygenase 2

CRP – C reactive protein

DIO – Diet induced obesity

DPI- Diphenylene iodonium

dsDNA- double stranded DNA

ERK- Extracellular signal-regulated kinase

GLUT4- Glucose transporter type 4

GTT - Glucose tolerance test

HFD – High fat diet

HMDM – Human monocyte derived macrophages

HOMA-IR – Homeostatic model assessment of insulin resistance

IAM – Iodoacetamide

IκBα – Inhibitor of nuclear factor kappa B alpha

IKKβ - IκB kinase beta

IL-1b- Interleukin 1 beta

IL-4 – Interleukin 4

IL-6 – Interleukin 6

IL-13 – Interleukin 13

IR – Insulin resistant

IRS-1 - Insulin receptor substrate 1

IS – Insulin sensitive

ITAM – Immunoreceptor tyrosine based activation motif

JNK - c-Jun N-terminal kinase

M1- Classical activation

M2- Alternative activation

MCP-1- Monocyte chemoattractant protein 1

MFI – Mean fluorescence intensity

MMe – Metabolic activation

NFkB – Nuclear factor kappa B

NAC- N-acetyl cysteine

NGS - Normal goat serum

NOX2 – NADPH oxidase 2

OGTT – Oral glucose tolerance test

PH – pleckstrin homology

PI3K - Phosphoinositide 3 kinase

PLCγ2 - Phospholipase C gamma 2

PLIN2 – Perilipin-2

PX – Phox homology domain

ROS – Reactive oxygen species

SOCS-3 – Suppressor of cytokine signaling-3

SVC – Stromovascular cells

SYK – Spleen tyrosine kinase

TCEP - tris(2-carboxyethyl)phosphine

TIR - Toll/interleukin-1 receptor

TLR – Toll-like receptor

TLR2 – Toll-like receptor 2

TLR4 – Toll-like receptor 4

TNF-α – Tumor necrosis factor alpha

TZD – Thiazoladinedione

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ABSTRACT

Insulin resistance and type 2 diabetes have reached epidemic levels in the United States. Mouse models have demonstrated that the increase in inflammatory cytokines during obesity is a mechanistically linked to insulin resistance. Adipose tissue macrophages, which accumulate and become activated during obesity, are a major source of this inflammation. These macrophages were often thought to be classically activated. However, studies of adipose tissue macrophages in humans demonstrate that the human obese adipose tissue macrophage phenotype is less straightforward. Our lab demonstrated that these macrophages exhibit a phenotype distinct from classical activation and redefined them as metabolically activated. Here we investigate the association of surface markers of metabolic activation with insulin resistance in humans. We use flow cytometry and a host of other parameters to show that insulin resistance is associated with omental ABCA1 expression independent of changes in inflammation. We also present an unexpected and strong relationship between ABCA1 expression and omental adipocyte size. These findings reveal a novel marker associated with insulin resistance in humans and raise further questions about role of adipose tissue macrophages in this disease. In our second aim we present a series of tyrosine kinases that promote metabolic activation. We also demonstrate that metabolic activation engages the transcription factor NFκB using a mechanism that is distinct from canonical NFκB signaling. These findings provide new therapeutic targets to treat insulin resistance through attenuating macrophage activation. Overall, this work provides insight into the metabolic activation phenotype at multiple levels and establishes a foundation to pursue the pathways that drive it.

CHAPTER ONE: COMPREHENSIVE INTRODUCTION

Pathophysiology of insulin resistance and type 2 diabetes

Insulin is a small peptide hormone synthesized in the β-cells of the pancreas in response to a post-prandial rise in blood sugar [1]. It binds to insulin receptors in an endocrine fashion and initiates the movement of glucose transporter type 4 (GLUT4) to the plasma membrane, allowing glucose to move from the blood into tissue. While many cell types have insulin receptors, the three major sinks for glucose disposal is the muscle, liver, and adipose tissue[1]. Prolonged overnutrition can lead to insulin resistance through multiple mechanisms including free fatty acid interference with insulin receptor substrate (IRS-1) in muscle and similar cytokine interference in the liver and adipose tissue[2,3]. β-cells respond to insulin resistance by increasing their insulin output and causing a state of hyperinsulinemia to control blood glucose levels. If unmanaged, insulin resistance can progress to type 2 diabetes, in which the elevated insulin levels can no longer compensate for the degree of insulin resistance[1]. Type 2 diabetes is characterized by both hyperinsulinemia and hyperglycemia and can lead to micro vessel damage, kidney failure, blindness and amputation[4]. As of 2018 almost 10% of patients in the United States have been diagnosed with type 2 diabetes. The prevalence of type 2 diabetes and the long term health risks associated with it make it one of the major public health burdens in the United States[4].

Historical associations between inflammation and insulin resistance

The first evidence for a relationship between inflammation and insulin resistance came from Dr. W. Ebstein in Gottingen [5]. Ebstein found treating patients with high doses of sodium salicylate reduced glucosuria in diabetic patients[6]. Twenty-five years later, in 1902, Dr. R.T. Williamson reported a case in which he used aspirin to treat a patient with glycosuria. While small doses for short times had not worked in previous cases, treating an inpatient with higher doses, 260mg/day, led to a marked reduction in the glucose excreted in the urine[7]. Decades later, Dr. James Reid reported a case of a 26 year old male controlling his blood sugar with insulin before being admitted to the hospital with rheumatic fever. Upon treatment with aspirin for the rheumatic fever it was found that he no longer required insulin to control his blood sugar. After discharge and discontinuation of the aspirin treatment he was readmitted to the hospital one week later with glycosuria[8]. Further studies continued to confirm the association between salicylic acid as an anti-diabetic agent[9,10]. However, there was no mention of its function as an anti-inflammatory agent. The first mention of salicylic acid's anti-inflammatory properties in relation to diabetes came from Norbiato and Foa in 1978, in which patients with diabetes were treated with aspirin and included other anti-inflammatory drugs (ibuprofen and ketoprofen) as a control[11]. At the end of the twentieth century salicylic acid's use as a cardioprotective agent quickly outpaced interest in its use as an insulin sensitizing agent[12]. However, more recent clinical trials have confirmed early associations between insulin resistance and salicylic acid as an anti-inflammatory agent[13–17].

While the anti-inflammatory properties of salicylic acid in diabetes went relatively unremarked, the link between inflammation and insulin resistance began to grow in the mid-twentieth century. Evidence of alterations in acute phase reaction proteins and a diminished capacity for fibrinolysis became associated with diabetes [18–21]. Levels of serum complement, both CH50 and C3 were found to be higher in patients with diabetes than their normoglycemic controls[22,23]. Interestingly, in 1980 it was shown that multiple components of the complement pathway (C3, C3 activator, and C4) were elevated in patients with diabetes and patients with glucose intolerance [24]. More evidence of the link between inflammation and insulin resistance came as reports of insulin resistance associated with traumatic inflammatory conditions. In 1974 John Kinney's group at Columbia compared the glucose tolerance a group of non-septic control patients to ones that had developed sepsis after injury. They found that the fasting blood sugar of the septic patients was significantly higher and they disposed of a glucose bolus less efficiently than healthy controls [25]. A group at Mt. Sinai, while investigating the response to endotoxin in type 1 diabetic patients, found that both the diabetics and healthy controls developed fasting hyperglycemia and insulin resistance in response to the endotoxin challenge[26]. Finally, Douglas Wilmore's team at Harvard used euglycemic clamps on patients recovering from non-septic trauma and showed that, in comparison to controls, they had reduced glucose disposal rates and elevated insulin resistance [27].

Adipose tissue as a target of proinflammatory cytokines

This clear complementary relationship between inflammation and insulin resistance led to a wave of studies in the 1990s and early 2000s that mechanistically connected inflammatory cytokines to insulin resistance. Tumor necrosis factor alpha (TNF-α) was the first cytokine to be mechanistically linked to impaired insulin receptor signaling. Bruce Spiegelman's group at Harvard published a series of studies beginning by demonstrating that antagonizing elevated TNF-α in insulin resistant mice improves their glucose disposal rate[28]. They then showed that TNF-α inhibits critical tyrosine phosphorylation and promotes inhibitory serine phosphorylation on the insulin receptor and IRS-1 respectively in liver, muscle, and adipocyte models[3,29,30]. The associations between TNF-α and insulin resistance were confirmed in humans with obesity and insulin resistance[31].

Another important cytokine, interleukin 6 (IL-6), which is associated with insulin resistance in humans, was shown to enhance expression of Suppressor of Cytokine Signaling-3 (SOCS-3). SOCS-3 can directly bind to the insulin receptor to impair autophosphorylation and IRS-1 phosphorylation in hepatocytes[32]. In an adipocyte model, Ulf Smith's group demonstrated that IL-6 impairs the expression of GLUT4 and IRS-1 expression and phosphorylation[33]. A third cytokine, interleukin 1 beta (IL-1β), was demonstrated to downregulate the expression of IRS-1 in both mouse and human adipocytes in an extracellular signal-regulated kinase (ERK) dependent manner[2]. Taken together, inflammatory cytokines have been well connected to impaired insulin signaling in muscle, liver, and fat.

In addition to the direct effects of inflammatory cytokines on insulin signaling in adipocytes, inflammation can alter the dynamics of adipocyte turnover to promote insulin resistance. In humans, average adipocyte size is associated with the incidence of metabolically unhealthy obesity and as early as 1968 it was shown that smaller isolated adipocytes had a greater response to insulin than larger adipocytes [34–39]. Preventing adipocyte hypertrophy in obesity requires the expansion of a population of stem cells into mature adipocytes to produce hyperplasia[40]. Studies have demonstrated that metabolic syndrome is associated with impaired differentiation of preadipocytes[41–43]. Inflammation has also been shown to inhibit the differentiation of stem cells into committed preadipocytes[44–48]. These data make a compelling story for a secondary mechanism by which inflammation promotes insulin resistance.

At the end of the twentieth century the relationship between systemic inflammation and insulin resistance became clear from both and mechanistic and epidemiological perspective. However, as the bulk of literature on inflammation concerns infection, the source of the inflammation was still unclear in a sterile environment. Using measures of C-reactive protein (CRP), an acute phase protein released by the liver in response to inflammation, researchers were able to recapitulate previous data showing a relationship between inflammation and insulin resistance. However, they also demonstrated that there was no relationship between titers of common infectious pathogens (*H. pylori*, *C. pneumoniae*, and cytomegalovirus) and either inflammation or insulin resistance in their cohort, confirming that obesity associated inflammation is not driven by infectious agents[49]. This group did find that adipose mass was well-correlated to inflammation and further studies demonstrated a clear relationship between fat mass, particularly trunk fat, and inflammation[49,50].

Macrophages as a source of inflammation during obesity

Adipose tissue mass had been documented as a major source of inflammation during obesity[49,50]. However, adipose tissue is a complex environment containing many cell types and the cell type responsible for obesity-associated inflammation was not yet clear. In 2003 Anthony Ferrante Jr.'s group at Columbia published a seminal study demonstrating that adipose tissue macrophages (ATMs) are a major contributor to inflammation[51]. Macrophages had previously been shown to infiltrate into adipose tissue to clear dead adipocytes during tissue injury and one group demonstrated an increase in dead adipocyte clearance during hypothalamic obesity[52]. Using multiple models of obesity and multiple adipose depots Ferrante's group demonstrated that transcript levels of macrophage specific genes (*Cd68* and *Csf1r*) were upregulated in the adipose tissue of obese mice. Immunohistochemical investigation revealed an increase in F4/80+ cells, a common macrophage marker, in obese fat. These F4/80+ cells were later confirmed to be ATMs via gene expression analysis of FACS-isolated cells and were shown to occupy nearly 50% of the cellular content of fat during obesity compared to 10% in the lean state[51].

Infiltrating adipose tissue macrophages were also shown to take on an inflammatory phenotype during obesity[51,53]. In Weisberg et al. they found that, while some inflammatory genes (*Il6*) are expressed equally across adipocytes and ATMs, genes including *Tnfa* and *Inos* are almost exclusively expressed in ATMs. Xu et al. also found an increase in inflammatory and macrophage specific genes in obese adipose tissue[53]. Of note they also showed that this increase in inflammation preceded a rise in fasting insulin levels. These inflammatory genes were also suppressed in response to the insulin sensitizing drug rosiglitazone.

Like mice, humans demonstrate increased adipose tissue macrophage infiltration during obesity[51,54–56]. Using FACS analysis, Curat et al. were able to show an increase in CD14+ cells in obese patients and that those CD14+ cells had higher expression of *MCP1*, *MIP1A*, and *IL8* [54]. Within only obese subjects, adipose tissue macrophage accumulation is associated with *TNFA* expression, hyperinsulinemia and insulin resistance[57]. Moreover, treatment with pioglitazone, an insulin sensitizer, significantly decreased *CD68* mRNA expression in adipose tissue; a proxy for macrophage infiltration[55].

Macrophage inflammation is linked to insulin resistance in mice *in vivo*

As *in vivo* associations in humans and *in vitro* mechanistic evidence of a link between ATM inflammation and insulin resistance grew, the question of causality remained. In 2008 Patsouris et al. identified a population of infiltrating macrophages in obese adipose tissue (F4/80+CD11b+CD11c+) that constituted the majority of the macrophage increase during obesity[58]. Using CD11c-diptheria toxin receptor mice they were able to selectively ablate this macrophage population in mice on high fat diet (HFD) and saw an amelioration of insulin resistance. Preventing the recruitment of macrophages into the tissue had a similar effect when a group in Japan knocked out monocyte chemoattractant protein 1 (MCP-1) in adipocytes and found reduced insulin resistance and hepatosteatosis[59].

Investigators also targeted macrophage derived cytokines to probe their effects on insulin resistance. In 2007 a group from Vanderbilt demonstrated that a bone marrow specific knockout of *Tnfa* was sufficient to protect mice from obesity induced insulin resistance[60]. Inhibiting IL-1β secretion, through inhibiting the NLRP3 inflammasome, protects mice on HFD from insulin resistance and hepatosteatosis [61].

In addition to perturbing the extracellular effectors of inflammation, investigators sought to identify the plasma membrane receptors responsible for initiating the events responsible for producing them. Toll-like receptors (TLRs) are a group of pattern recognition receptors that are able to recognize molecular characteristics broadly associated with microbial pathogens[62,63]. The Hwang group at the Western Human

Nutrition Research Center published a series of papers demonstrating fatty acids can initiate inflammation and that this requires toll like receptors (TLRs). They demonstrated that RAW264.7 cells, a murine macrophage-like cell line, increase expression of the inflammatory enzyme cyclooxygenase-2 (COX2) in response to saturated fatty acids (lauric and palmitic acid) in a toll like receptor 4 (TLR4) dependent manner[64,65]. They also showed that this pathway was dependent on TLR4 homodimerization and classical TLR4 signaling proteins including MyD88, IRAK-1, TRAF6, and IκBα[66,67]. Additionally, they showed that toll-like receptor 2 (TLR2) is also able to mediate COX2 and IL-1β expression in human 293T cells and mouse bone marrow derived macrophages through heterodimerization with TLR1 or TLR6[67,68]. Other groups have shown similar results, suggesting that the ability of TLRs to detect bacterial associated lipid moieties is repurposed in lipid-rich environments to respond elevated free fatty acids[69,70].

In mouse models, free fatty acid (oleate and palmitate) infusion induces insulin resistance in muscle that is ameliorated in *Tlr4* knockout mice. In a diet-induced obesity model *Tlr4* knockout was also protective against insulin resistance and inflammation in multiple models[71,72]. Multiple studies have demonstrated bone marrow specific *Tlr4* knockout mice had less adipocyte and stromovascular expression of *Tnfa* and *Il6* and improved insulin sensitivity on high fat diet[73,74]. In the case of TLR2, whole body knockout mice had reduced inflammation and improved insulin sensitivity on HFD[75–78].

In addition to extracellular receptors, many groups have targeted established intracellular inflammatory signaling pathways in macrophages to probe their effects on insulin resistance. One of the primary inflammatory signaling pathways in macrophage is the canonical NFκB pathway. This pathway culminates in the activation of IκB kinase β (IKKβ) which phosphorylates and targets NFκB's inhibitor for degradation; leaving NFκB free to enter the nucleus to promote the expression of proinflammatory genes[79]. In 2005 Jerrold Olefsky's group made a myeloid-specific knockout of *Ikkb* that was protected from systemic insulin resistance on high fat diet challenge as measured by glucose tolerance tests (GTT) and euglycemic-hyperinsulinemic clamp studies [80]. Because the liver inflammation is a major contributor to systemic insulin resistance, they also made a similar knockout in hepatocytes[80,81]. While the hepatocyte-specific knockout improved hepatic insulin resistance, it did not show the systemic improvements observed in the myeloid-specific knockout mouse[80]. Other studies have demonstrated that disrupting the NFκB pathway protects against inflammation and insulin resistance[71]. A second inflammatory signaling pathway relies on a c-Jun N-terminal kinase (JNK) which enters the nucleus and phosphorylates the transcription factor c-Jun, allowing it to heterodimerize into an active complex[82]. Macrophage-specific *Jnk1/2* knockout mice and recipients of *Jnk-/-* bone marrow on high fat diet had reduced adipose inflammation and improved glucose and insulin tolerance[83,84]. Taken together, these studies demonstrate that disrupting macrophage inflammation at multiple levels is protective against insulin resistance in mice.

Reciprocally, targeting macrophage pathways that suppress inflammation exacerbates insulin resistance. The discovery of these relationships begins with the development of thiazoladinediones (TZDs). In the 1980s TZDs were reported to have insulin sensitizing properties and in the 1990s they were discovered to be an agonist of a nuclear receptor PPARγ[85,86]. Despite some controversy in the field because of the insulin sensitive phenotype of a *Ppargγ+/-* mouse, PPARγ became firmly established as an insulin sensitizing pathway, primarily through its action on adipocyte development and triglyceride loading[87–89]. In addition to its role in adipocyte biology, PPARγ was also shown to influence macrophage inflammation. PPARγ agonists were shown to inhibit inflammatory cytokine release *in vitro*[90–92]. In mouse models of DIO, myeloid-specific *Pparg* knockout mice had increased ATM inflammation, impaired glucose disposal, impaired insulin action in the muscle and liver, and reduced response to TZD treatment[93–95]. By the 2010's, macrophage inflammation had been firmly established as both necessary and sufficient for the development of insulin resistance in mice.

Macrophages were discovered in the context of host defense

Macrophages were described in the early 1900's in Elie Metchnikoff's Immunity in Infection Disease as a cell characterized by "phagocytosis, mobility, and high metabolic production"[96]. Much of the twentieth century was dedicated to describing what is now termed "classical activation" of macrophages and their role in pathogen killing[97]. In the late 1980's and early 1990's multiple investigators showed that Th2 cytokines such as interleukin 4 (IL-4) and interleukin 13 (IL-13) were able to inhibit the proinflammatory cytotoxic activation of macrophages[98–104]. In 1992 Stein et al. demonstrated IL-4 treatment upregulated expression of the macrophage mannose receptor and posited that, rather than inhibiting macrophage activation, IL-4 was initiating an anti-inflammatory alternative activation pathway[105]. More evidence for an alternative pathway emerged in 1994 when Doyle et al. demonstrated that IL-13 also suppressed TNF-α but upregulated macrophage mannose receptor expression [106]. Thus emerged the classical/alternative activation paradigm of macrophage activation.

Classical activation (M1) is characterized by microbial killing, reactive oxygen species (ROS) production, and inflammatory cytokine release[107]. In contrast, alternative activation (M2) promotes angiogenesis, tissue repair, and is anti-inflammatory[107,108]. Applying this paradigm to adipose tissue macrophages produced a model in which ATMs maintain an M2 phenotype in the lean state but adopt an M1 phenotype during obesity[109–111]. Obese adipose tissue macrophages do not fit perfectly in the category of classical activation because they have certain characteristics that are typical of alternative activation. These include expression of surface proteins characteristic of M2 macrophages in mice (CD206, CD163), upregulation of M2-associated genes (*Arg1, Mmp12, Vegf*) and M2 functions including tissue remodeling and angiogenesis[112–114].

In 2014 the Becker lab showed that obese ATMs were not, in fact, classically activated[115]. Using plasma membrane proteomics, they established consistent markers of classical activation (CD319, CD274, CD38) and demonstrated that these markers were absent on obese adipose tissue macrophages[115]. Rather, obese ATMs showed elevated expression of unique surface proteins including ATP binding cassette subfamily A member 1 (ABCA1), perilipin 2 (PLIN2), and fatty acid translocase (CD36) that correlate with body mass index (BMI)[115,116]. Like classical activation, these metabolically activated macrophages (MMe) express inflammatory cytokines but to a lesser degree than M1 macrophages[115]. In a subsequent paper we inhibited metabolic activation with a myeloid-specific *Nox2* knockout. *Nox2-/-* mice showed reduced inflammation and insulin resistance during diet-induced obesity (DIO) despite being heavier than their wildtype counterparts. We also found that metabolic activation played a crucial role in the resolution of crown-like structures and the clearance of dead adipocytes [78,117]. While metabolic activation clearly plays an important role in obesity, much of the biology of metabolic activation is unknown.

The relevance of metabolic activation in human insulin resistance is unknown

It is well-established that macrophage infiltration is associated with obesity in humans[51,54–56]. Previous work in the Becker lab has also shown the MMe phenotype also associates with obesity[115,116]. However, obesity alone is not inherently dangerous. Patients with obesity fall on a broad spectrum of metabolic health and can generally be classified as having metabolically healthy or metabolically unhealthy obesity. The definition of metabolically unhealthy obesity varies but generally refers to patients with insulin resistance, elevated blood pressure, or other components of metabolic syndrome[118,119]. Metabolically unhealthy obesity is much more dangerous than metabolically healthy obesity and is associated with an elevated risk of all-cause mortality compared to healthy obese controls[120,121]. Insulin resistance specifically is associated with an elevated risk for cardiovascular disease and incidence of 6 different types of cancer[122,123]. While work from Coats et al. showed that metabolic activation contributes to insulin resistance in mice, there is no data on the relationship between metabolic activation and insulin resistance in humans[78]. This dissertation will address this question.

Metabolic activation requires NADPH oxidase 2 (NOX2)

Previous work in the Becker lab showed that metabolically activated inflammation in macrophages is driven by the fatty acid palmitate and relies on TLR2 and NOX2[78,115]. We also presented evidence to suggest that, while metabolic inflammation was attenuated, classical inflammation remained intact in *Nox2* knockout bone marrow derived macrophages (BMDMs)[78]. This early evidence suggests that metabolic activation utilizes a different inflammatory signaling pathway than classical activation. This dissertation will present evidence in support of a novel pathway and its role in metabolic activation of macrophages.

CHAPTER TWO: METABOLIC ACTIVATION OF ADIPOSE TISSUE MACROPHAGES IS ASSOCIATED WITH INSULIN RESISTANCE IN HUMANS

Introduction

More than 1.9 billion adults worldwide are overweight or obese[124]. Obesity is a well-established risk factor for insulin resistance and type 2 diabetes, which affects more than 451 million people worldwide and is associated with an increased risk of both cardiovascular events and cancer[120–123,125–127].

Insulin resistance in humans is associated with inflammation and macrophage accumulation in adipose tissue. These macrophages become activated and inflamed during obesity but their phenotype does not fit into the established M1/M2 paradigm as they have both M1 and M2 characteristics and lack well-established markers including iNOS and arginase [128,129]. Moreover, despite the strong connection between macrophage inflammation and insulin resistance in mice, anti-inflammatory therapies in humans have been largely unsuccessful in treating insulin resistance. These contradictions highlight the pressing need for a deeper understanding of adipose tissue macrophage biology during obesity and insulin resistance in humans.

Our lab redefined these obese ATMs as metabolically activated, a phenotype that associates with BMI in humans and has shared characteristics of both M1 and M2. While inflamed, these obese adipose tissue macrophages also upregulate lysosomal pathways and fatty acid oxidation which is typical of anti-inflammatory macrophages [130,131]. In addition to inflammation, MMe ATMs upregulate proteins involved in lipid processing including ABCA1, PLIN2, and CD36 [78,115]. They rely heavily on their lysosomal system to process apoptotic adipocytes and maintain healthy adipose tissue [78,117]. Metabolic activation, is driven by free fatty acids engaging with TLRs; an interaction which other groups have also reported[67,68,78]. In mice, we have used myeloid-specific MMe knockouts to demonstrate the contribution of MMe inflammation to both insulin resistance and cancer stemness[78,116]. This integrated model for obese adipose tissue macrophage biology can inform our understanding of metabolic disease and give rise to innovative therapeutics.

We have shown metabolic activation of visceral adipose tissue macrophages associates with BMI in humans[115,116]. However, the relationship between metabolic activation and insulin resistance in humans is unknown. To answer this question, we have assembled a cohort of patients with obesity and use a combination of flow cytometry and immunofluorescence to interrogate multiple depots within each patient to evaluate the relationship between metabolic activation and insulin sensitivity. Using this new macrophage phenotype as a tool we aim to better understand the biology of obese adipose tissue macrophages. This understanding is desperately needed and can lead to more effective anti-inflammatory therapies and preventative measures in the treatment of insulin resistance.

Materials and Methods

Ethics- This study was approved by the Institutional Review Board at Fred Hutchinson Cancer Research Center (IRB#8340).

Subject recruitment- Patients with obesity undergoing elective bariatric surgery were recruited from the Puget Sound Surgery Center in Edmonds, WA. Exclusion criteria include smoking, alcohol/drug abuse, pregnancy, history of cardiovascular disease, autoimmunity, other chronic inflammatory conditions, use of anti-diabetic or anti-inflammatory drug, steroids, or hormones.

Anthropomorphic measurements- Body fat percentage was measured using a Dual-Energy X-Ray Absorptiometry Scan (iDXA-scanner, GE Healthcare). Liver fat was measured by fMRI.

Oral glucose tolerance test- After fasting and an initial blood draw, subjects drank 75g of glucose and blood was sampled at 10, 20, 30, 60, 90, 120, and 180 minutes post-surgery. 5mL of blood was sampled at each time point.

Blood measurements- High sensitivity CRP, glucose, insulin, IL1β, IL6, TNFα, and MCP1 were measured by enzyme linked immunosorbent assay (ELISA) at Northwest Lipid Research Laboratories.

Adipose tissue collection and processing- 5-10g of abdominal subcutaneous and omental adipose tissue was collected. Small pieces ~100mg were snap frozen or paraffin fixed. Adipose stromovascular cells (SVC) were isolated using a 60 min collagenase I digestion as described [132].

Histological staining and analysis- For adipocyte sizing, paraffin-fixed samples were stained for perilipinA (Abcam, Cambridge UK). 5 slides per subject were stained and imaged on a Nikon Eclipse Ti2 at 10x and 5 randomly selected fields were taken per slide. Analysis was done using a skeletonize thresholding method and the area in microns^2 was measured. For crown-like structure (CLS) quantification, paraffin-fixed samples were stained for Mac-2 (Cedarlane CL8942AP). 5 slides per subject were stained and imaged and the entire tissue area was scanned at 10x. CLS quantification was done by hand with the definition of a CLS as Mac-2+ signal that encompasses at least 180 degrees of an adipocyte. CLS counts were normalized to total tissue area.

Whole cell proteomics- Mass spectrometric analyses and statistical analyses of proteomics data were performed as previously described[133].

FACS Analysis- Cells were sorted on an LSR-II from BD Biosciences (San Diego. CA). Dendritic cells were gated as CD14+ CD206+ CD1c+ CD11c+ and neutrophils as CD15+ CD16^{hi}[134–138]. After gating for dendritic cells and neutrophils adipose tissue macrophages were defined as CD14+ CD206+[134]. Antibodies used were purchased from BD Biosciences (Franklin Lakes, NJ), Beckman Coulter (Danvers, MA), Novus Biologicals (Littleton, CO) BioLegend (San Diego, CA), eBioscience (San Diego, CA), or Miltyeni (Auburn, CA).

Flow cytometry analysis- SVC from lipedema lipoaspirate was classified as immune cells (CD45+), macrophages (CD45+ CD11b+ CD14hi), or neutrophils (CD45+ CD11b+, CD16hi) with anitbodies purchased from BD Biosciences (Franklin Lakes, NJ). For MMe marker quantification macrophages were defined as CD11b+ CD14+.

Adipose tissue conditioned media (Ad-CM) treatment- whole adipose tissue was minced and incubated 24 hours in RPMI with 10% FBS and penicillin-streptomycin. Peripheral blood monocytes were isolated from healthy donors and differentiated into human monocyte derived macrophages (HMDMs) using macrophage colony stimulating factor (M-CSF). HMDMs were treated with Ad-CM for 24 hours and RNA was extracted using a Qiagen RNeasy Micro Kit.

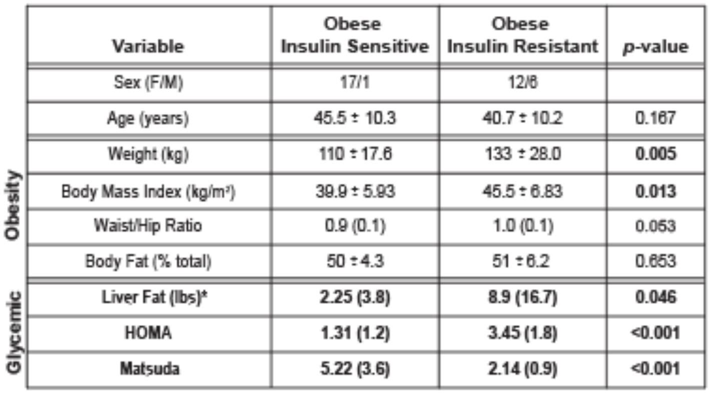
Statistical Analysis- All variables were checked for normal distribution using Shapiro-Wilk tests. Those that were normally distributed were presented as mean +/-

SD and those that were non-normally distributed are presented as median (interquartile range). In the cases where insulin sensitivity is treated as a categorical variable all non-normally distributed variables were log transformed before using a t-test. In cases where insulin sensitivity is treated as a continuous variable sex and age were adjusted for as assumed confounders based on literature. All other potential confounders were checked for association with both insulin sensitivity and marker expression before being adjusted for. A *p*-value of $p<0.05$ was considered significant.

Results

Participants are obese adults with a spectrum of insulin sensitivity

While MMe ATMs correlate with obesity in humans, the relationship between MMe ATMs and insulin sensitivity remains unknown. To investigate this relationship, we recruited patients undergoing bariatric surgery at Eviva Bariatric Services. Patients with a BMI greater than 30 kg/m^2 were recruited and categorized as insulin sensitive (IS, HOMA-IR<2.5) or insulin resistant (IR, HOMA-IR>2.5) based on their Homeostatic



	Variable	Obese Insulin Sensitive	Obese Insulin Resistant	*p*-value
	Sex (F/M)	17/1	12/6	
	Age (years)	45.5 ± 10.3	40.7 ± 10.2	0.167
Obesity	Weight (kg)	110 ± 17.6	133 ± 28.0	**0.005**
	Body Mass Index (kg/m^2)	39.9 ± 5.93	45.5 ± 6.83	**0.013**
	Waist/Hip Ratio	0.9 (0.1)	1.0 (0.1)	0.053
	Body Fat (% total)	50 ± 4.3	51 ± 6.2	0.653
Glycemic	**Liver Fat (lbs)***	**2.25 (3.8)**	**8.9 (16.7)**	**0.046**
	HOMA	**1.31 (1.2)**	**3.45 (1.8)**	**<0.001**
	Matsuda	**5.22 (3.6)**	**2.14 (0.9)**	**<0.001**

Figure 2.1 Patient Demographics. Normally distributed variables (Mean SD), skewed variables (median (interquartile range)). *P* values determined by two-tailed t-test after Levene's test for equivalence of variances. Non-normally distrbuted variables were log-transformed before performing a Student's t-test. **n= 10 IS/8 IR.*

Model Assessment on Insulin Resistance (HOMA-IR) score[139]. There was no significant difference in age between the IS (45.5 ± 10.83) and IR (40.7 ± 10.2) groups. While all participants are obese, the mean BMI of the IR group (45.5 ± 6.83 kg/m^2) was slightly, but significantly, higher than the IS group (39.9 ± 5.93 kg/m^2) (**Figure 2.1**). However, BMI only considers the height and weight of an individual, conflating lean and fat body mass. The ratio of lean to fat body mass, body fat percentage, is more predictive of health outcomes and associates with risk of type 2 diabetes[140,141]. Participants had no significant difference in body fat percentage between the IS (50 ± 4.3) and IR (51 ± 6.2) groups (**Figure 2.1**).

To assess insulin sensitivity, subjects underwent fasting glucose and insulin measurements in addition to an oral glucose tolerance test (OGTT). There was no significant difference in fasting glucose between the IS (95 ± 6 mg/dL) and IR (98 ± 9 mg/dL) groups (**Figure 2.2A**). However, fasting insulin was elevated in the IR group (16.7 ± 7.3 μU/mL) compared to IS group (5.8 ± 2.5 μU/mL) (**Figure 2.2A**). Similarly, OGTT glucose AUC was unchanged between groups while OGTT insulin AUC was elevated in the IR group (17143 ± 5738 mg/dLxmin) compared to the IS group (8466 ±

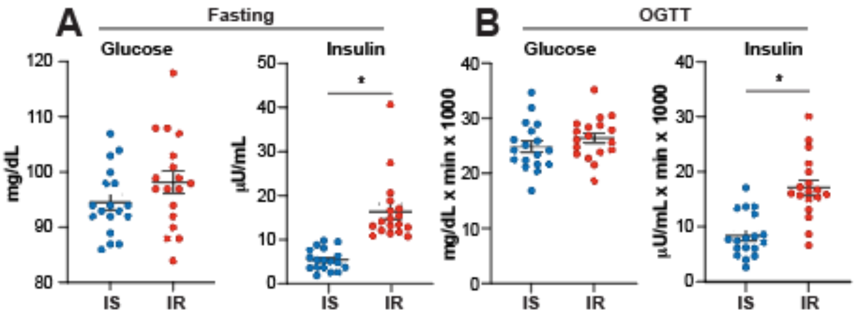


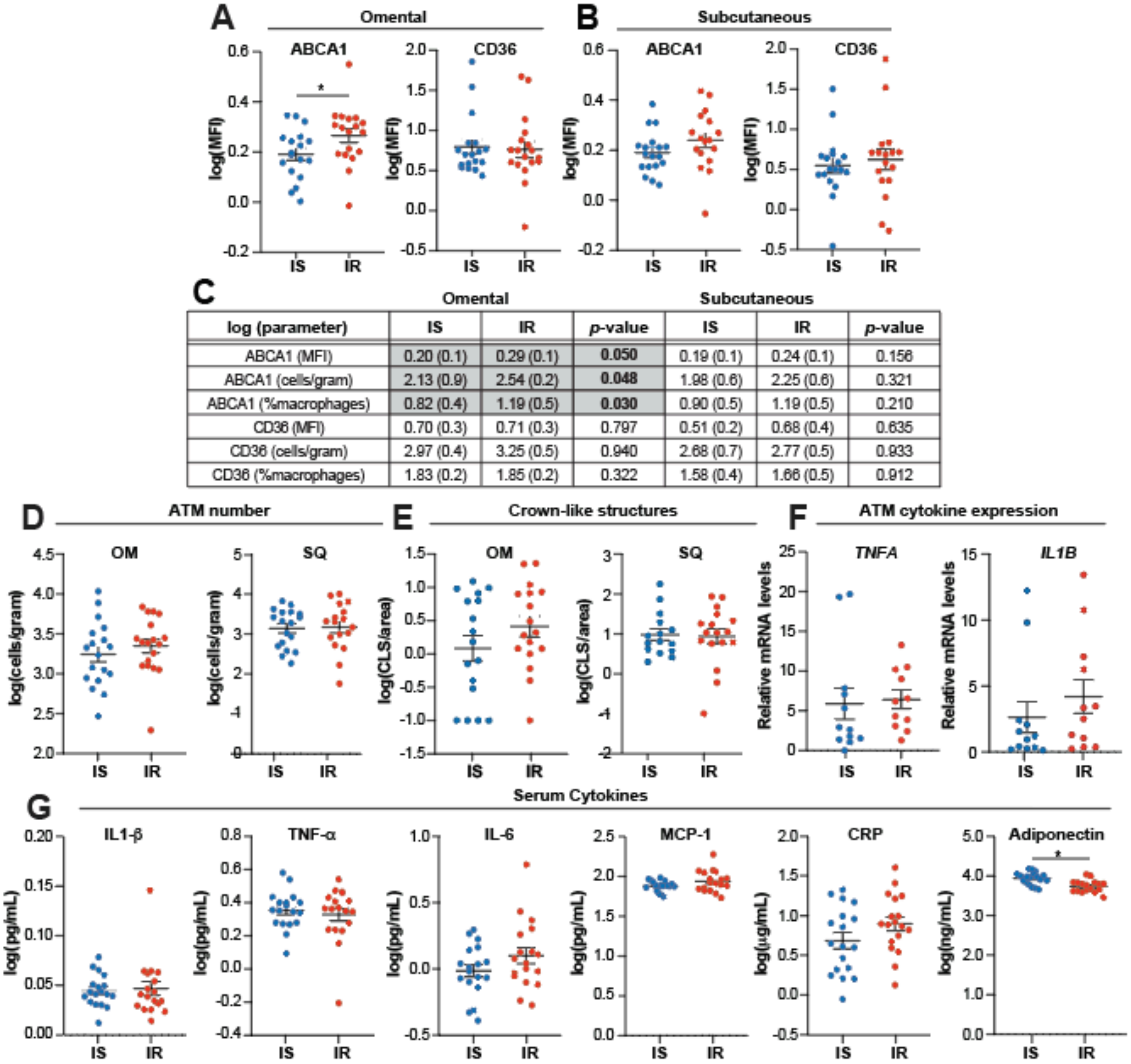
Figure 2.2 Cohort glycemic control. (A) Fasting glucose and insulin (*n=18*) (B) Glucose and insulin area under the curve during oral glucose tolerance test (OGTT) (*n=18*). * $p<0.05$ Student's t-test

3970 mg/dLxmin) (**Figure 2.2B**). Collectively, these data indicate that the IR group is significantly insulin resistant, but not hyperglycemic.

MMe-like ATMs are elevated in IR patients in the absence of changes in inflammation

MMe macrophages secrete inflammatory cytokines and upregulate surface proteins related to lipid processing including ABCA1 and CD36 [115]. We interrogated both the surface expression of MMe markers (mean fluorescent intensity, MFI) and the abundance of MMe marker positive cells (positive cells/gram tissue, and positive macrophages/total macrophages). Previous studies show that ABCA1 and CD36 expression are elevated in obese omental and subcutaneous adipose relative to lean controls[115]. Both ABCA1 expression and the abundance of ABCA1+ cells were elevated in omental ATMs in the IR group (**Figure 2.3A,C**). In contrast, there was no significant difference in ABCA1 expression or frequency of ABCA1+ cell in the subcutaneous depot (**Figure 2.3B,C**). CD36 showed no difference between groups in either the omental or subcutaneous depot (**Figure 2.3A-C**).

Insulin resistance is closely associated with both systemic and adipose tissue inflammation[142,143]. ATMs are a major source of this inflammation and obesity is associated with increased ATM number, formation of crown-like structures, and inflammatory activation[51,53,144,145]. We evaluated the inflammatory status of patients using several approaches. First, we quantified ATMs (CD14+ CD206+ CD1c- CD11c- CD15-), and found no difference in abundance between groups in both omental or subcutaneous adipose (**Figure 2.3D**). Second, crown-like structures showed no difference between IR and IS groups in either depot (**Figure 2.3E**). Third, macrophages isolated from both depots had no significant difference in expression of inflammatory cytokines *TNFA* and *IL1B* between groups (**Figure 2.3F**). Similarly, systemic inflammatory IL-1β, TNF-α, IL-6, MCP1, and CRP levels were unchanged (**Figure**

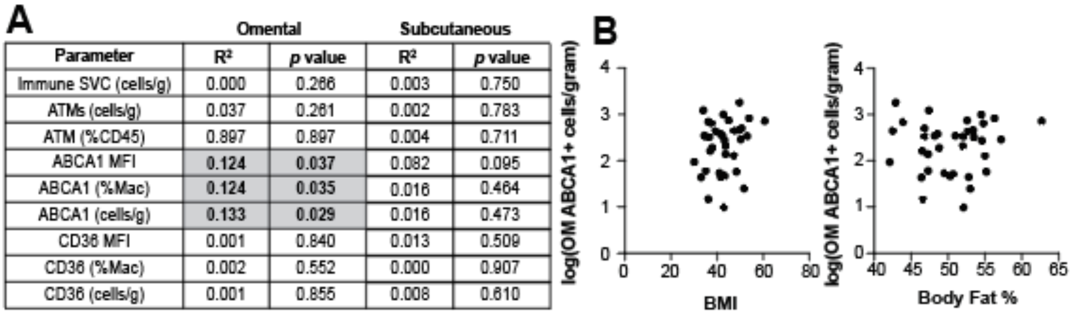


	Omental			Subcutaneous		
log (parameter)	IS	IR	*p*-value	IS	IR	*p*-value
ABCA1 (MFI)	0.20 (0.1)	0.29 (0.1)	**0.050**	0.19 (0.1)	0.24 (0.1)	0.156
ABCA1 (cells/gram)	2.13 (0.9)	2.54 (0.2)	**0.048**	1.98 (0.6)	2.25 (0.6)	0.321
ABCA1 (%macrophages)	0.82 (0.4)	1.19 (0.5)	**0.030**	0.90 (0.5)	1.19 (0.5)	0.210
CD36 (MFI)	0.70 (0.3)	0.71 (0.3)	0.797	0.51 (0.2)	0.68 (0.4)	0.635
CD36 (cells/gram)	2.97 (0.4)	3.25 (0.5)	0.940	2.68 (0.7)	2.77 (0.5)	0.933
CD36 (%macrophages)	1.83 (0.2)	1.85 (0.2)	0.322	1.58 (0.4)	1.66 (0.5)	0.912

Figure 2.3 MMe-like ATMs are elevated in IR patients in the absence of changes in inflammation. (A) Mean fluorescent intensity (MFI) of ABCA1 and CD36 from omental and (B) subcutaneous ATMs. (C) Quantification via MFI, cells/gram, and percent of macrophages of ATM ABCA1 and CD36 in IS and IR patients. (D) ATM (CD14+ CD206+ CD1c- CD11c- CD15- CD16lo) quantification in omental (OM) and subcutaneous (SQ) adipose.(E) Immunohistochemistry was used to identify Mac-2+ crown like structures which were divided by total tissue area. (F) mRNA expression of inflammatory cytokines from FACS sorted omental ATMs. (G) Serum inflammatory cytokines quantified with ELISA.* $p<0.05$ Student's t-test

2.3G). Together, these findings show that inflammation is not elevated in the IR patients.

Omental ATM ABCA1 associates with insulin resistance

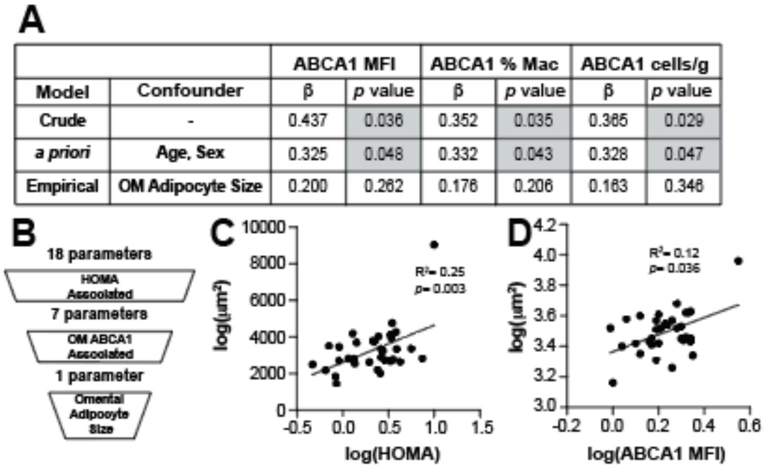


	Omental		Subcutaneous	
Parameter	R^2	*p* value	R^2	*p* value
Immune SVC (cells/g)	0.000	0.266	0.003	0.750
ATMs (cells/g)	0.037	0.261	0.002	0.783
ATM (%CD45)	0.897	0.897	0.004	0.711
ABCA1 MFI	**0.124**	**0.037**	0.082	0.095
ABCA1 (%Mac)	**0.124**	**0.035**	0.016	0.464
ABCA1 (cells/g)	**0.133**	**0.029**	0.016	0.473
CD36 MFI	0.001	0.840	0.013	0.509
CD36 (%Mac)	0.002	0.552	0.000	0.907
CD36 (cells/g)	0.001	0.855	0.008	0.610

Figure 2.4 Omental ABCA1 associates with insulin resistance. (A) Associations of immune, macrophage, and MMe with HOMA-IR in omental and subcutaneous adipose tissue. (B) Associations betwen ABCA1 and metrics of obesity.

We then explored ABCA1's association with insulin resistance using linear regression models. In a basic linear regression, participants showed a significant association between insulin resistance and omental ATM ABCA1 expression and abundance despite a lack of association between insulin resistance and immune stromovascular cells or ATM accumulation (**Figure 2.4A**). Neither CD36 expression nor frequency showed significant correlation to insulin resistance in either depot (**Figure 2.4A**). Importantly, omental ABCA1 specifically correlates with insulin resistance and does not associate with obesity as measured by body fat percentage or BMI (**Figure 2.4B**).

We next tested if this relationship was independent of 18 potential confounding variables. Sex and age were adjusted for *a priori* based on the strong association with insulin resistance in the literature[146,147]. Adjusting for sex and age did not attenuate the relationship between omental ATM ABCA1 and insulin resistance (**Figure 2.5A**). Among



		ABCA1 MFI		ABCA1 % Mac		ABCA1 cells/g	
Model	**Confounder**	β	*p* value	β	*p* value	β	*p* value
Crude	-	0.437	0.036	0.352	0.035	0.365	0.029
a priori	**Age, Sex**	0.325	0.048	0.332	0.043	0.328	0.047
Empirical	**OM Adipocyte Size**	0.200	0.262	0.176	0.206	0.163	0.346

Figure 2.5 The association between ABCA and HOMA-IR is attenuated by omental adipocyte size. (A) Linear regression models of the relationship between ABCA1 and HOMA-IR. (B) Schematic of empirical confounder selection. (C) Linear regression of omental adipocyte size and HOMA-IR. (D) Linear regression of omental adipocyte size and ABCA1.

the eighteen potential confounders considered, the association between ABCA1 expression of omental ATMs and insulin resistance was independent of BMI, age, liver fat content, visceral adipose volume, systemic inflammation, and circulating adiponectin (**Figure 2.5B**). However, omental adipocyte size strongly associated with both HOMA-IR and the omental ATM ABCA1 (**Figure 2.5C,D**). Inclusion of omental adipocyte size in the model strongly attenuated the relationship between insulin resistance and our measures of ABCA1 expression on omental ATMs (**Figure 2.5A**).

Omental adipose tissue from IR patients increases *ABCA1* expression in macrophages

To investigate a possible mechanistic link between omental adipocyte size and ABCA1 we used collected adipose tissue in an organ culture system (**Figure 2.6A**). We previously showed that Ad-CM collected from obese patients was better able to drive the MMe phenotype in HMDMs than Ad-CM from lean patients[115]. In this *ex vivo* organ

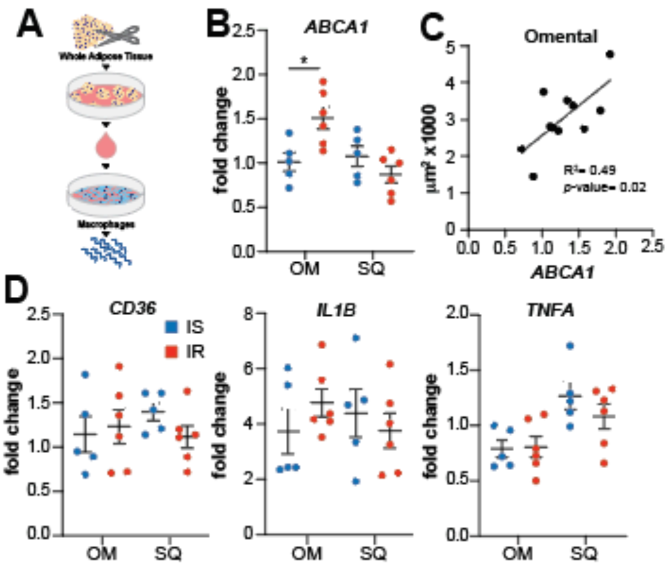


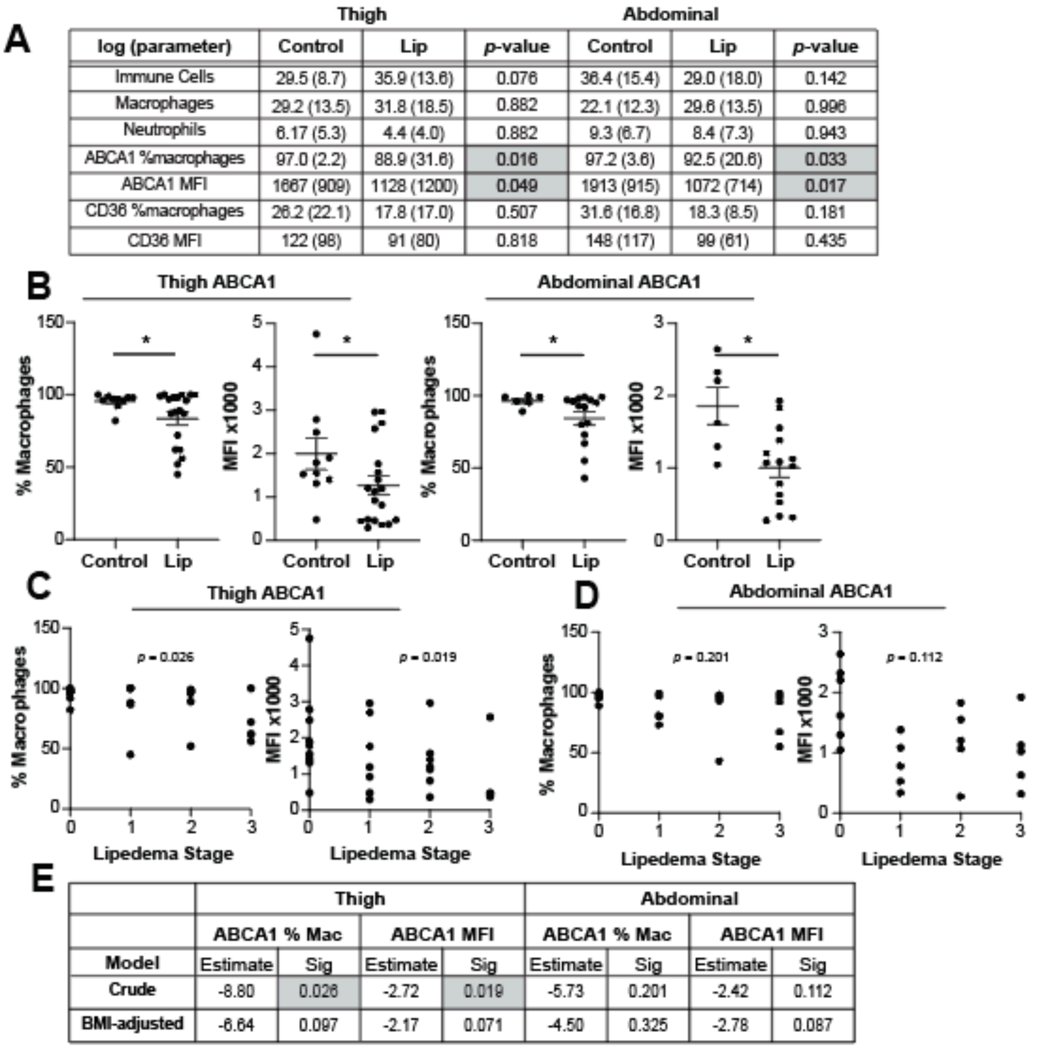
Figure 2.6 Omental adipose tissue from IR patients increases ABCA1 expression in macrophages. (A) Schematic of adipose tissue conditioned media (Ad-CM) collection and treatment. (B) ABCA1 mRNA expression in Ad-CM treated macrophages. (C) Linear regression of ABCA1 mRNA expression against subject's average omental adipocyte size. (D) mRNA expression of MMe markers *CD36*, *IL1B*, and *TNFA*.* $p<0.05$ Student's t-test

culture system, expression of *ABCA1* was elevated in macrophages treated with omental Ad-CM from the IR group when compared to the IS group (**Figure 2.6B**). Expression of *ABCA1* in the macrophages treated with omental Ad-CM also correlated with the patient's average omental adipocyte size (**Figure 2.6C**).

Consistent with previous figures, subcutaneous Ad-CM treated HMDMs displayed no difference in *ABCA1* expression between groups (**Figure 2.6B**). Ad-CM mediated induction was specific to *ABCA1* as there was no change in *CD36* expression the IS and IR group in either depot (**Figure 2.6D**). Moreover, there was no significant difference in induction of inflammatory genes *TNFA* and *IL1B* between IS and IR groups in either depot (**Figure 2.6D**).

ATM ABCA1 in lipedema

In addition to obesity and insulin resistance, we investigated the expression of MMe markers in adipose tissue from patients with lipedema, a lipid disorder characterized by the painful accumulation of subcutaneous adipose tissue in the lower extremities[148]. Adipose tissue in lipedema shows increased micro vessel fragility, impaired lymphatic function, and expansion of adipose stem cell populations[149–151]. We



	Thigh			Abdominal		
log (parameter)	Control	Lip	*p*-value	Control	Lip	*p*-value
Immune Cells	29.5 (8.7)	35.9 (13.6)	0.076	36.4 (15.4)	29.0 (18.0)	0.142
Macrophages	29.2 (13.5)	31.8 (18.5)	0.882	22.1 (12.3)	29.6 (13.5)	0.996
Neutrophils	6.17 (5.3)	4.4 (4.0)	0.882	9.3 (6.7)	8.4 (7.3)	0.943
ABCA1 %macrophages	97.0 (2.2)	88.9 (31.6)	0.016	97.2 (3.6)	92.5 (20.6)	0.033
ABCA1 MFI	1667 (909)	1128 (1200)	0.049	1913 (915)	1072 (714)	0.017
CD36 %macrophages	26.2 (22.1)	17.8 (17.0)	0.507	31.6 (16.8)	18.3 (8.5)	0.181
CD36 MFI	122 (98)	91 (80)	0.818	148 (117)	99 (61)	0.435

	Thigh				Abdominal			
	ABCA1 % Mac		ABCA1 MFI		ABCA1 % Mac		ABCA1 MFI	
Model	Estimate	Sig	Estimate	Sig	Estimate	Sig	Estimate	Sig
Crude	-8.80	0.026	-2.72	0.019	-5.73	0.201	-2.42	0.112
BMI-adjusted	-6.64	0.097	-2.17	0.071	-4.50	0.325	-2.78	0.087

Figure 2.7 ATM ABCA1 in lipedema. (A) Median and interquartile range of immune parameters in thigh and abdominal lipoaspirate. (B) ABCA1 number (%macrophages) and expression (MFI) (C) Thigh ABCA1 number and expression vs lipedema stage (D) Abdominal ABCA1 number and expression vs lipedema stage (E) Ordinal linear regression models of ABCA1 and lipedema stage.

used flow cytometry to probe the MMe markers ABCA1 and CD36 in subcutaneous thigh and abdominal lipoaspirates. Comparing lipedema to non-lipedema patients showed no difference in immune cell, macrophage, or neutrophil accumulation **(Figure 2.7A).** Despite no difference in macrophage number, we found that ABCA1 expression in lipedema patients was significantly lower than control in both the thigh and abdominal samples **(Figure 2.7B)**. There was no significant difference in CD36 in either depot **(Figure 2.7A)**. Examining ABCA1 relative to the stage of the patient disease showed an inverse association between ABCA1 expression and lipedema stage in the thigh tissue**(Figure 2.7C)**. There was also a similar trend in the abdominal tissue **(Figure 2.7D)**. Because we've previously shown a positive relationship between ABCA1 and obesity, we chose to adjust for BMI in our model comparing ABCA1 and lipedema stage. Adjusting for BMI slightly attenuated the relationship but maintained a trend of an inverse relationship between ABCA1 and lipedema stage **(Figure 2.7E)**. While this data reflects our obese cohort in that we see little change in immune infiltration, it inverts the relationship between ABCA1 and disease severity identified in insulin resistance.

Discussion

Decades of evidence show adipose tissue macrophage inflammation contributes to insulin resistance. Mouse studies suggest that this inflammation is caused by a transition from an anti-inflammatory M2 ATM phenotype to proinflammatory M1 during obesity[145]. However, human ATM phenotypes during obesity are less clear and can have both M1 and M2 characteristics[113,128,152]. Our lab previously described a novel macrophage phenotype, metabolic activation, that has both the inflammatory characteristics of M1 macrophages, and the lipid metabolism of M2 macrophages [115].

MMe activation associates with obesity in humans but its relationship to insulin resistance is unknown. Importantly, obesity and insulin resistance are complex diseases that are associated with a host of changes such as immune activation, fat accumulation, ectopic fat deposition, and adipose tissue dysfunction. To fully characterize the relationship between insulin resistance and MMe we measured more than 20 parameters in each subject over a series of clinical visits prior to bariatric surgery when fat samples were collected. The extensive nature of this study made it extremely difficult to recruit non-obese patients. Here we provide evidence that the MMe marker ABCA1 in omental ATMs is associated with insulin resistance. We also found this relationship is reliant on omental adipocyte size, specific to omental adipose tissue macrophages, and independent of all but one potential confounder.

Multiple metrics of inflammation indicate that there was no elevation in inflammation in the IR group over that observed in the IS group. First, there was no significant difference in systemic cytokines CRP, TNF-α, IL-1β, IL-6, CRP, or MCP1 between the IS and IR groups. At the tissue level, there was no increase in macrophage abundance or crown like structure formation. Finally, there was no change in the expression of inflammatory cytokines *TNFA* or *IL1B* in isolated ATMs. This is in contrast to other studies that report positive associations between inflammation and insulin resistance [142,153,154]. While many of these studies also recruit obese individuals, the mean BMI of these cohorts is often <33kg/m^2. The mean BMI of our IS and IR group is 39.9+/-5.9 and 45.5 +/- 6.8, respectively. Other studies of cohorts with similarly high BMIs show elevated inflammation, macrophage abundance, and macrophage polarization in obese subjects compared to lean controls but no differences within the obese groups associated with insulin sensitivity[155–158]. A possible explanation of the lack of difference in inflammation is that this study was not designed to include a patient's timeline of disease. Mouse studies indicate that inflammation can precede the long-term development of insulin resistance[53]. Thus, our insulin sensitive group may actually represent a pre-insulin resistant group that has not yet accumulated enough inflammation-related damage to impair their insulin response. Longitudinal studies support this notion and show metabolically healthy obese patients have a higher chance (OR= 3.13) of progressing to type 2 diabetes than non-obese controls[159]. Additionally, an American group found of the 85 metabolically healthy obese patients recruited, 55 (65%) progressed to metabolically unhealthy obesity over the 10 observation period[160]. Based on this evidence, it's possible that inflammation alone is insufficient to predict insulin resistance and must be integrated into a broader understanding of the timeline of the patient's disease.

Omental adipose tissue accumulation is closely associated with insulin resistance in humans and the expansion of this depot is correlated with increases in systemic inflammation. Compared to the subcutaneous depot, ATMs in the visceral depot are more inflamed during diet induced obesity[53]. While we saw no differences in inflammation, multiple lines of evidence indicate the relationship between ABCA1 and insulin resistance is also restricted to the omental depot. First, ABCA1 was elevated in ATMs in the IR group in the omental depot but not the subcutaneous depot. Linear regressions also showed a robust positive association between ATM ABCA1 and insulin resistance in the omental depot but none in the subcutaneous depot. Finally, only Ad-CM from omental fat was able to drive *ABCA1* expression *ex vivo*. Taken together, these data suggest that omental adipose tissue is uniquely able to drive the expression of ABCA1 in adipose tissue macrophages.

The ability of the omental adipose tissue to drive *ABCA1* expression seems to be dependent on adipocyte size. Omental adipocyte size was the only parameter that associated with both insulin resistance and omental ATM ABCA1. When included in a regression model with HOMA-IR and omental ATM ABCA1 omental adipocyte size attenuated their positive relationship. Furthermore, the ability of omental Ad-CM to drive *ABCA1* expression *ex vivo* was positively associated with the average adipocyte size. In humans, larger adipocytes are more insulin resistant on an individual level [35]. Insulin suppresses lipolysis and insulin resistant adipocytes have higher levels of lipolysis and free fatty acid release. Our previous work demonstrated that free fatty acids, specifically palmitate, can drive the MMe phenotype *in vitro* [115]. Taken together, these findings implicate insulin resistance and lipolysis associated with larger adipocytes as a driver of ABCA1 expression in ATMs.

Interestingly, our second surface marker of metabolic activation, CD36, did not associate with insulin resistance. Previous work on the MMe phenotype demonstrated both *Abca1* and *Cd36* expression was driven *in vitro* by palmitate and their surface expression on ATMs correlated with BMI[115]. Compared to lean patients Ad-CM from obese patients was also able to drive both *ABCA1* and *CD36* expression *ex vivo*[115]. Because we were unable to recruit lean subjects we must rely on these previous studies and their conclusion that CD36 expression is associated with obesity. In contrast, there was no difference in CD36 expression or abundance between IS and IR patients. There was also no difference in IR Ad-CM's ability to drive *CD36* expression compared to IS.

Combined, these data suggest that CD36 is primarily associated with obesity rather than insulin resistance. Taking into account the model that MMe is a fatty-acid driven phenotype, the lack of association between insulin resistance and CD36 could be due to a difference in the lipid species released during obesity versus insulin resistance. Other groups have shown changes in as many as 10 fatty acids species associated with insulin resistance in obese individuals [161,162]. These differences in lipid species may drive different components of the MMe phenotype.

Here we show a robust and specific relationship between omental ATM ABCA1 and insulin resistance. ABCA1 has been shown to dampen inflammation in macrophages *in vitro*[163–165]. Myeloid *Abca1* knockout mice in a diet induced obesity model have elevated adipose tissue macrophage accumulation, inflammation, and are insulin resistant[166]. These data taken together suggest ATM ABCA1 is protective during obesity. This raises the possibility that omental ATMs are also playing a protective role and ABCA1 elevation in insulin resistant patients is an attempt to reestablish homeostasis. We have already shown MMe activation plays a protective role during obesity by promoting the clearance of dead adipocytes[78]. Others have shown macrophage activation can regulate lipolysis during fasting and is required for the healthy expansion of adipose tissue[167,168]. Thus, ABCA1 elevation may be another example of a beneficial role of activated adipose tissue macrophages during obesity.

A potential mechanism of ABCA1's protective function is its ability to modify inflammatory pathways. ABCA1's cholesterol export function cell allows it to modify membrane composition[169]. These alterations in membrane composition, specifically at lipid rafts, can dampen inflammatory signaling in macrophages[163,164]. ABCA1's anti-inflammatory capacity may also contribute to the lack of difference in inflammation between the IS and IR groups. These data provide a potential new marker of metabolic dysfunction in adipose tissue macrophages. Understanding the biological role of ABCA1 in ATMs during insulin resistance may provide new insights into the role of ATMs in the pathophysiology of insulin resistance.

CHAPTER THREE: A SERIES OF TYROSINE KINASES MEDIATE METABOLIC INFLAMMATION

Introduction

Inflammation is closely associated with insulin resistance [143]. Mouse models have allowed the field to mechanistically show the detrimental effects of inflammatory cytokines on the insulin signaling pathway [3,30]. Attenuating obesity associated inflammation, by either depletion of inflamed ATMs or inhibition of macrophage inflammatory pathways, can preserve insulin sensitivity during diet induced obesity [58,73]. This evidence led to the pursuit of anti-inflammatory therapies as a treatment for insulin resistance in humans.

Unfortunately, translating these results into humans been largely ineffective. Neutralizing TNF-α, the earliest cytokine connected to insulin resistance, had no effect on insulin sensitivity in human subjects and only a modest effect on fasting glucose after 6 months of treatment [170–174]. IL-1β blockade shows some improvement in HbA1c levels but no improvements in insulin sensitivity[175–179]. Even salicylates, which have shown the most clinical efficacy in improving glucose disposal are thought to act by increasing insulin output rather than sensitivity [13–17]. Since improving insulin sensitivity by targeting a single inflammatory product has not proved successful, a better understanding of the source of this inflammation could provide a new target that cuts inflammation off at the source rather than targeting its products.

Adipose tissue macrophages are a major source of obesity-related inflammation. During obesity, macrophages accumulate in adipose tissue and become inflamed [51,53].

This inflammation was originally seen as a shift from an anti-inflammatory M2 phenotype in the lean state to an inflamed M1 phenotype in the obese state [145]. Proteomic interrogation revealed obese ATMs are not classically activated but adopt a unique metabolically activated inflammatory phenotype characterized by moderate inflammation (IL-1β, IL-6, TNF-α) and upregulation of proteins associated with lipid processing (ABCA1, CD36, PLIN2) (**Figure 3.1**)[115]. Inhibiting metabolic activation in a mouse model of diet induced obesity decreased adipose tissue inflammation and improved glucose tolerance despite increased body weight[78]. These data suggest that metabolic activation is a promising target for obesity driven inflammation. Our own data shows that TLR2, TLR4, NOX2, and MyD88 are required for metabolic activation[78]. However, the mechanism by which these proteins produce metabolic inflammation, and more specifically, how they differ from classical activation are unknown.

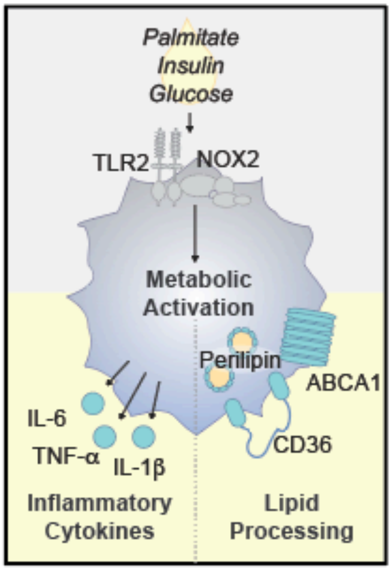


Figure 3.1 Palmitate, insulin, and glucose induce metabolic activation using TLR2 and NOX2.

Here we use a combination of proteomic and hypothesis driven approaches to interrogate the signaling pathways that give rise to metabolic activation. Beginning with the investigation of Nuclear factor-κB (NFκB), a common transcription factor in inflammatory signaling, we demonstrate that NFκB activity is required for metabolic activation. However, we also show that the dynamics and participants in NFκB activation in MMe macrophages are distinct from M1 macrophages: suggesting a distinct NFκB activation pathway for MMe macrophages. We then use our finding of NOX2 as a critical contributor to MMe signaling to identify ROS as a second messenger. We go on to identify a target of this second messenger and reveal a role for two tyrosine kinases rarely associated with macrophage biology. These data provide critical evidence of a new pathway of macrophage activation and a potential new target for anti-inflammatory therapies in obesity.

Materials and Methods

Isolation and activation of bone marrow derived macrophages- Hematopoietic cells were flushed from mouse femurs and tibias and cultured in 30% L-cell conditioned media for 6 days as previously described[115]. Macrophages were metabolically or classically activated on day 7 of differentiation using 0.4mM palmitate, 580.8ng/mL insulin, and 0.54% glucose or with 12ng/mL interferon gamma and 5ng/mL lipopolysaccharide. Unless otherwise specified, cells for RT-PCR were activated for 6 hours and cells for immunofluorescence were activated for 2 hours.

Inhibitors- Cells treated with inhibitors Diphenyleneiodonium (3μM), N-acetyl cysteine (1mM), JSH-23 (25μM), U-73122 (5μM) and Entospletinib (8μM) were pretreated for 1 hour before activation and the inhibitors were present in the activation media. Cells treated with the inhibitors Ibrutinib (25μM) and Piceatannol (25μM) were pretreated for 30 minutes. All inhibitors were maintained in media during the 2 or 6 hour activation. Inhibitors were purchased from SelleckChem (Houston, TX) or Sigma (St. Louis, MO).

Quantitative Real Time Polymerase Chain Reaction- RNA was isolated using a Qiagen RNeasy mini prep kit and converted to cDNA with Qiagen's reverse transcription kit. Genes are normalized to 18s and quantified using the $2^{-\Delta\Delta Ct}$ method[180]. All primers were purchased from Integrated DNA Technologies (Coralville, IA).

Immunofluorescence- Cells were grown in 8 chamber slides. After activation, cells were fixed in a 10% formalin solution for 10 minutes and then permeabilized with PBS with 0.3% Triton. Samples were blocked with 5% normal goat serum (NGS) in PBST (0.02% Tween20) for 1 hour at room temperature. Primary antibodies (CST, Danvers MA) were diluted 1:250 in 1% NGS in PBST and incubated with the sample overnight at 4°C. Secondary antibodies were diluted 1:500 in 1% NGS in PBST and incubated at room temperature for 2 hours. Images were taken on a Nikon Ti2 Eclipse within 48 hours of staining.

Immunofluorescence Analysis- Images were analyzed in ImageJ. Background was removed by subtracting a minimum filtered image from the original with a kernel size of 25 pixels for DAPI channel and a kernel size of 50 pixels for all other channels. Regions of Interest were created for the nuclear and cytosolic areas using the Intermodes and Li autothreshold algorithms respectively. Signal was quantified as RawIntegratedDensity/Area and NFκB localization was represented as nuclear/cytosolic signal.

Western blot- Cells were lysed in 1% SDS supplemented with protease and phosphatase inhibitor. Protein concentration was quantified using a Pierce BCA Assay and 20ug of protein was loaded for each sample. Samples were run on a 12.5% gel and transferred onto a nitrocellulose membrane. Blots were blocked with either 5% milk or 5% phosphoblock for 1 hour at room temperature. Primary and secondary antibodies were diluted 1:1000 and developed using ECL western blotting substrate.

Biotin switch assay- macrophages were metabolically activated for 6 hours. Activated cells were collected in alkylating buffer (50mM Tris-HCl pH 8.5, 8M Urea, 5mM EDTA, 18.6mg/mL iodoacetamide) and incubated for 15 minutes. The samples were precipitated and washed with cold acetone and resuspended in reduction buffer (50mM Tris-HCl pH 8.5, 8M Urea, 5mM EDTA, 10 mM tris(2-carboxyethyl)phosphine) for 30 minutes. Samples were washed and labeled with labeling buffer (50mM Tris-HCl ph 8.5, 8M Urea, 5mM EDTA, 0.4mM biotin-HPDP EZlink) for 2 hours before quenching with 100mM cysteine. Labeled proteins were enriched on a streptavidin column and eluted with 50mM DTT. Samples were digested with 1:20 trypsin and cleaned on an SepPac column with acetonitrile and formic acid buffers.

Results

A novel activation of the NFκB pathway is required for metabolic activation of macrophages

Because both M1 and MMe macrophages express inflammatory cytokines (**Figure 3.2A**), we began exploring MMe inflammation using pathways established in M1 macrophages. NFκB is a transcription factor that controls the expression of many inflammatory genes in macrophages [181]. Olefsky's group have shown that the NFκB pathway is required for obesity related inflammation and subsequent insulin resistance in mouse models [80]. In classical activation, the p65 and p50 subunits of NFκB translocate to the nucleus upon activation [79]. To visualize NFκB's cellular localization in metabolic activation we used immunofluorescence with classical activation as a positive control. Images show both p65 and p50 are subunits excluded from the nucleus in the M0 unstimulated condition (**Figure 3.2B,C**). After 2 hours of MMe stimulation, both subunits translocate to the nucleus (**Figure 3.2B,C**). In contrast, NFκB translocation in

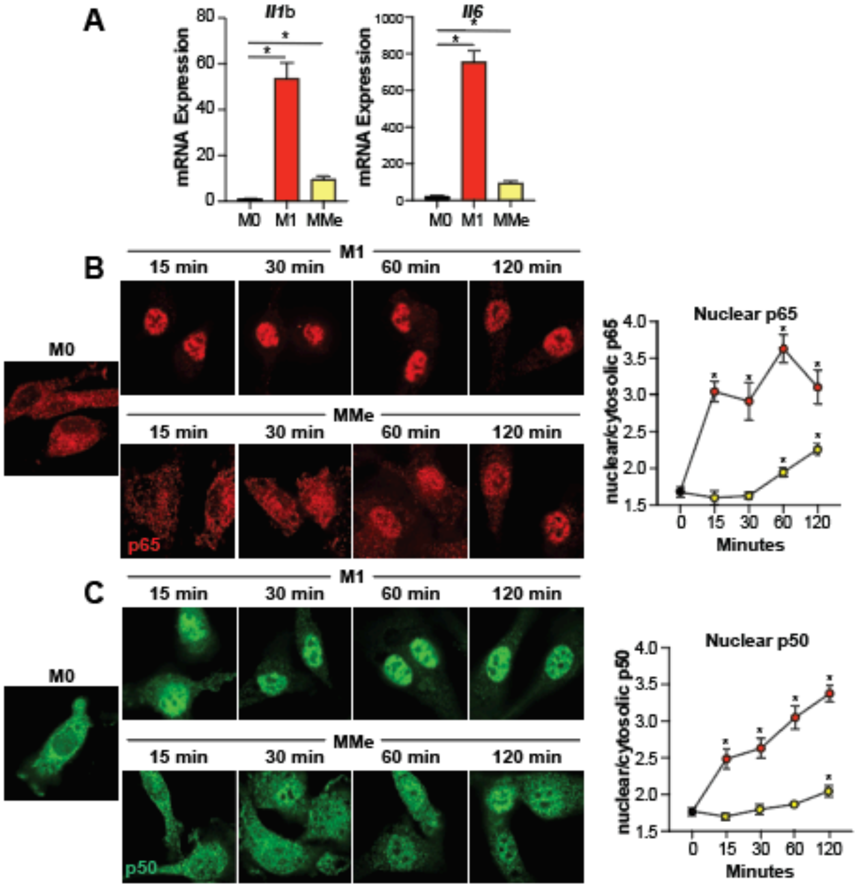


Figure 3.2 A novel activation of the NFκB pathway is required for metabolic activation of macrophages.(A) Cytokine levels in M0, M1, and MMe macrophages. (B) NFκB p65 nuclear translocation assessed by immunofluroescence. (C) NFκB p50 nuclear translocation assessed by immunofluroescence.

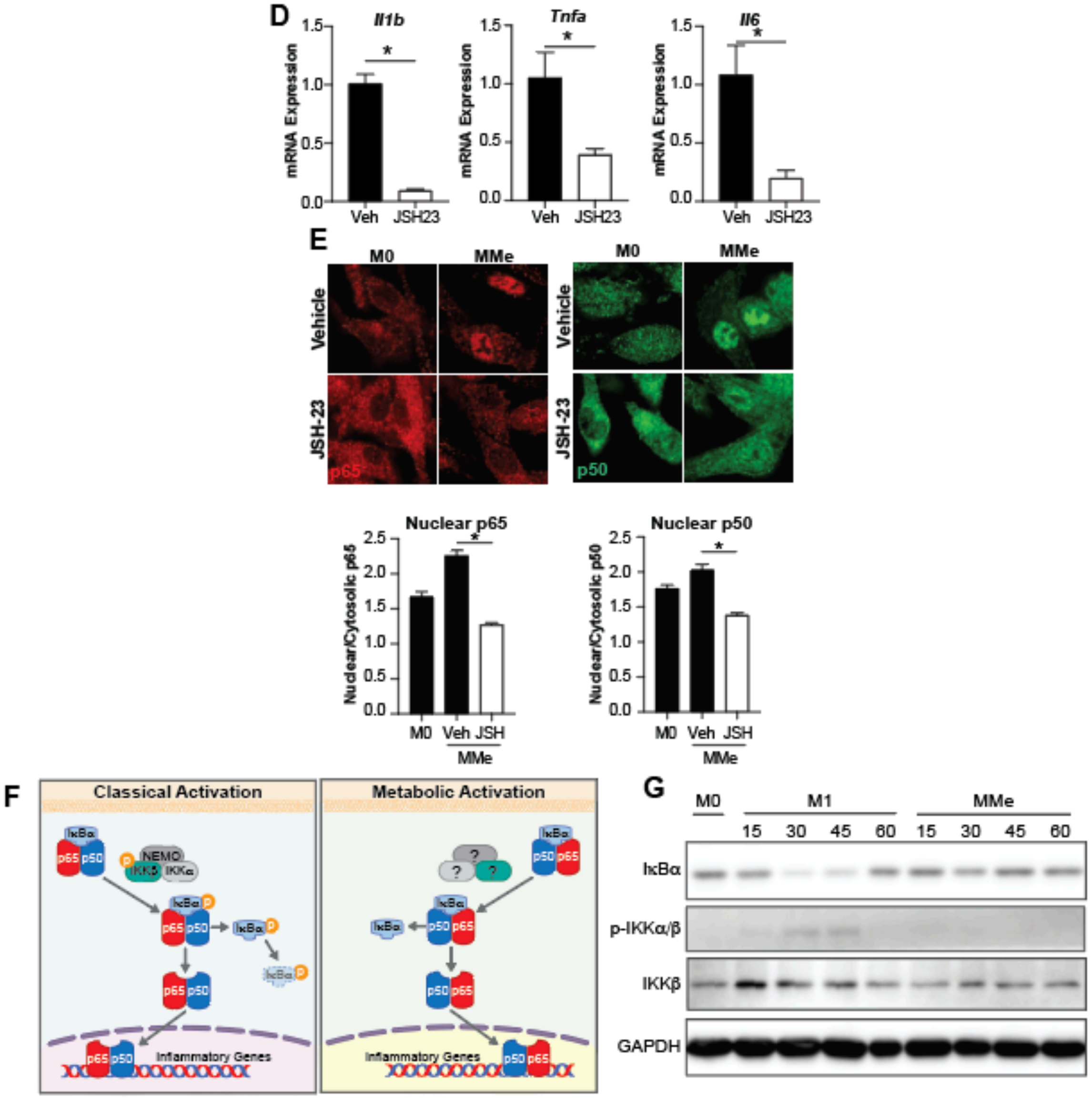


Figure 3.2 cntd (D) Macrophages pretreated with vehicle (Veh) or JSH-23 (25mM, p65 inhibitor) for 1 hour prior to and during MMe activation. mRNA levels are plotted relative to Veh for each group. (E) BMDMs treated with 25μM JSH-23 and activated for 2 hours. NFκB p65/p50 nuclear translocation assessed by immunofluroescence. (F) Existing model of M1 and MMe activation. (G) Western blot analysis of canonical NFκB signaling in M1 and MMe macrophages. Results are Mean and SEM.
* *p*<0.05 Student's t-test

M1 macrophages begins after 15 minutes of stimulation and continues to increase, peaking at 60 minutes (**Figure 3.2B,C**). Additionally, the extent of NFκB nuclear translocation was higher in M1 macrophages than MMe macrophages at every timepoint (**Figure 3.2B,C**). Although we show movement of NFκB to the nucleus in MMe macrophages, it's requirement for MMe activation is still unknown.

To determine if NFκB nuclear localization is required for metabolic activation, we treated BMDMs with JSH-23, an inhibitor of p65 nuclear translocation. BMDMs treated with JSH-23 show reduced translocation of NFκB to the nucleus, as expected, and attenuated induction of MMe genes *Il1b*, *Tnfa*, and *Il6* compared to vehicle controls (**Figure 3.2D**) (**Figure 3.2E**). Taken together, these data suggest MMe and M1 activation share the NFκB transcription factor. We then hypothesized that MMe activation also shares upstream regulators of NFκB with M1 activation.

In classical activation, both p65 and p50 subunits of NFκB are sequestered together in the cytosol by inhibitor of nuclear factor kappa B alpha (IκBα) [79]. Upon activation, IκBα is phosphorylated by IKKβ, leading to its degradation[79]. The degradation of IκBα allows NFκB to move to the nucleus where it activates inflammatory genes (**Figure 3.2F**). To probe these events, whole cell lysates of M1 and MMe BMDMs were probed for IKKβ phosphorylation and IκBα degradation. M1 BMDMs show phosphorylation of IKKβ and a demonstrable loss of IκBα (**Figure 3.2G**). In contrast, MMe macrophages show no phosphorylation of IKKβ or degradation of IκBα (**Figure 3.2G**). These data, in addition to the significantly slower and reduced NFκB nuclear translocation, suggest MMe activation does not require the canonical upstream components of NFκB signaling.

NOX2 produces reactive oxygen species that are required for metabolic activation

To pursue this pathway leading to NFκB signaling in macrophages, we first interrogated the initiating events of MMe activation. We previously showed that MMe activation requires NOX2, a membrane bound electron transport chain that resides on the plasma membrane and phagolysosome [78]. NOX2's electron transport chain produces superoxides that are rapidly converted to hydrogen peroxide and other ROS[182,183]. In host defense, this creates an oxidative burst within a phagolysosome for pathogen killing [183].

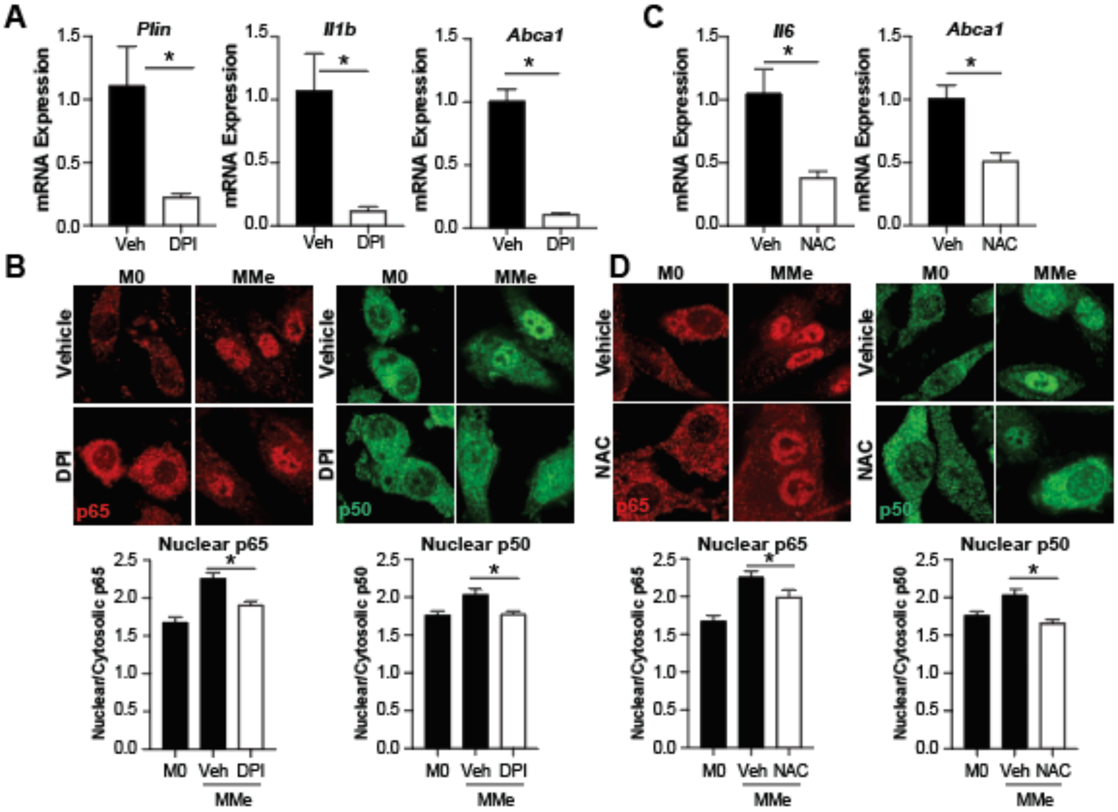


Figure 3.3 NOX2 produces reactive oxygen species that are required for metabolic activation(A) Macrophages were pretreated with vehicle (Veh) or DPI (3μM, Nox2 inhibitor) for 1 hour prior to and during MMe activation. mRNA levels are plotted relative to Veh for each group. (B) NFκB p65/p50 nuclear translocation assessed by immunofluorescence. (C) Macrophages pretreated with Veh or NAC (1mM, antioxidant). Levels plotted relative to Veh. (D) NFκB p65/p50 nuclear translocation assessed by immunofluroescence. * $p<0.05$ Student's t-test

To examine NOX2's contribution to the MMe phenotype BMDMs were treated with the NOX2 inhibitor diphenyleneiodonium (DPI). MMe BMDMs treated with DPI had significantly attenuated *Il1b*, *Abca1*, and *Plin* expression (**Figure 3.3A**). Additionally, DPI treatment reduced movement of NFκB to the nucleus (**Figure 3.3B**). To determine if NOX2-derived ROS are required for metabolic activation, BMDMs were treated with the ROS scavenger N-acetyl-cysteine (NAC). BMDMs treated with NAC had reduced *Il6* and *Abca1* upon MMe activation (**Figure 3.3C**). NAC also impaired NFκB nuclear translocation during MMe activation (**Figure 3.3D**).

Taken together, these results suggest that ROS participate in metabolic activation. Other groups have shown that ROS can both directly and indirectly modify signaling kinases[184]. Based on this context and our findings, we hypothesize that ROS are a secondary messenger in metabolic activation by modifying a downstream protein to change its function.

BTK is a target of NOX2 reactive oxygen species and required for metabolic activation

To identify potential targets of ROS in MMe macrophages we enriched for oxidized proteins with a modified biotin switch assay and use mass spectrometry to identify proteins oxidized following metabolic activation [185]. After activation, BMDMs were treated with iodoacetamide (IAM) to permanently block any unoxidized residues. Samples were then reduced in a species non-specific manner with tris(2-carboxyethyl)phosphine (TCEP) to reveal the previously oxidized groups. The newly revealed residues are then labeled with an IAM-conjugated biotin (**Figure 3.4A**). The biotin-conjugate proteins were enriched using a streptavidin column and run on LC-

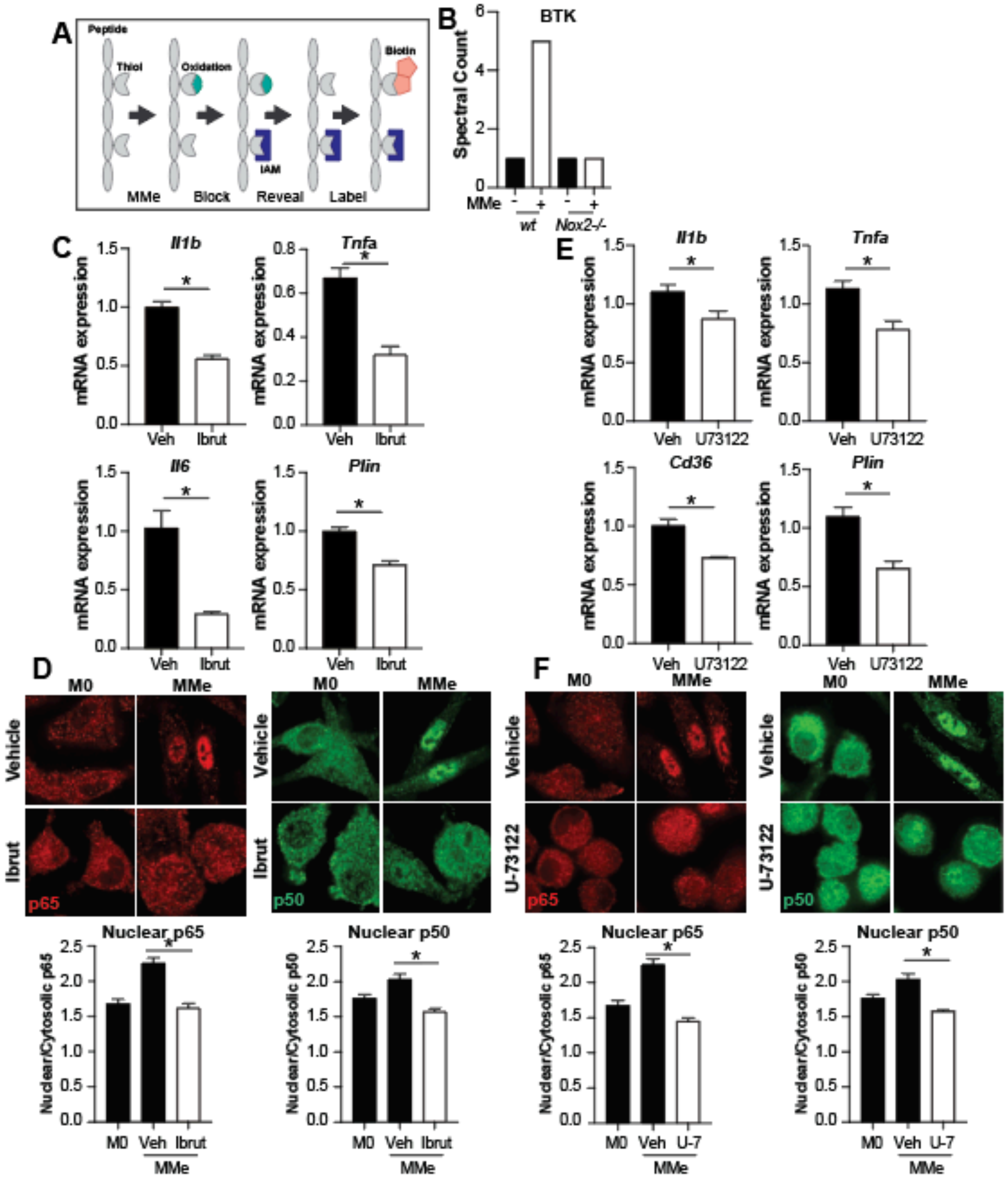


Figure 3.4 BTK is a target of NOX2 reactive oxygen species and is required for metabolic activation (A) Schematic of biotin switch assay (B) Counts of BTK eluted from streptavidin beads in *wt* and *Nox2-/-* BMDMs. (C) Macrophages were pretreated with vehicle (Veh) or Ibrutinib (Ibrut) (25μM, BTK inhibitor) for 30 minutes prior to and during MMe activation. mRNA levels are plotted relative to Veh for each group. (D) NFκB p65/p50 nuclear translocation assessed by immunofluorescence. (E) Macrophages pretreated with Veh or U-73122 (U-7) (5μM, PLCγ2 inhibitor). Levels plotted relative to Veh. (D) NFκB p65/p50 nuclear translocation assessed by immunofluroescence. * $p<0.05$ Student's t-test

MS/MS. To ensure specificity to NOX2-derived ROS, BMDMs from *Nox2-/-* mice were treated in parallel. This method identified Bruton's tyrosine kinase (BTK) as significantly enriched in wildtype MMe samples compared to M0 controls (**Figure 3.4B**). This enrichment was absent in *Nox2-/-* BMDMs, suggesting that the modification was specific to NOX2-derived ROS (**Figure 3.4B**).

BTK is a tyrosine kinase that interacts with immunoreceptor tyrosine based activation motifs (ITAM) on B cell receptors, and is essential for B cell development and activation [186]. It contains a pleckstrin homology (PH) domain that allows it to localize to the plasma membrane where it's typically phosphorylated and activated [187]. Knockout studies demonstrate BTK contributes to macrophage antibacterial and antiviral response through its interactions with TLRs [188–190]. BTK is reported to either interact with TLRs directly through a supposed Toll/interleukin-1 receptor (TIR) domain or by direct interaction and phosphorylation with the TIR-containing adaptor protein MAL/TIRAP[191,192]. In a promising study, administration of ibrutinib, a BTK inhibitor, improved obesity-associated inflammation in a mouse model of diet induced obesity[193]. While BTK has been shown to participate in LPS-stimulated macrophage activation, its contribution to MMe activation is unknown.

To investigate the contribution of BTK to metabolic activation, BMDMs were treated with ibrutinib before metabolic activation. Treatment with ibrutinib attenuated expression of *Il1b*, *Tnfa*, *Il6*, and *Plin*, suggesting that it is required for metabolic activation (**Figure 3.4C**). Moreover, BMDMs treated with ibrutinib had reduced nuclear translocation of NFκB compared to vehicle treated control (**Figure 3.4D**).

Once activated, BTK phosphorylates phospholipase C gamma 2 (PLCγ2) at tyrosine$^{753/759}$[194]. Activated PLCγ2 catalyzes the breakdown and release of signaling molecules to widely propagate a signal across the cell[195]. To assess PLCγ2's contribution to metabolic activation, BMDMs were treated with U-73122, a PLCγ inhibitor [196]. Treatment with U-73122 reduced the expression of *Il1b*, *Tnfa*, *Cd36*, and *Plin* in and impaired NFκB nuclear translocation in MMe macrophages (**Figure 3.4E,F**). Taken together, these data suggest that BTK is oxidized and activated by NOX2-derived ROS.

SYK contributes to metabolic activation

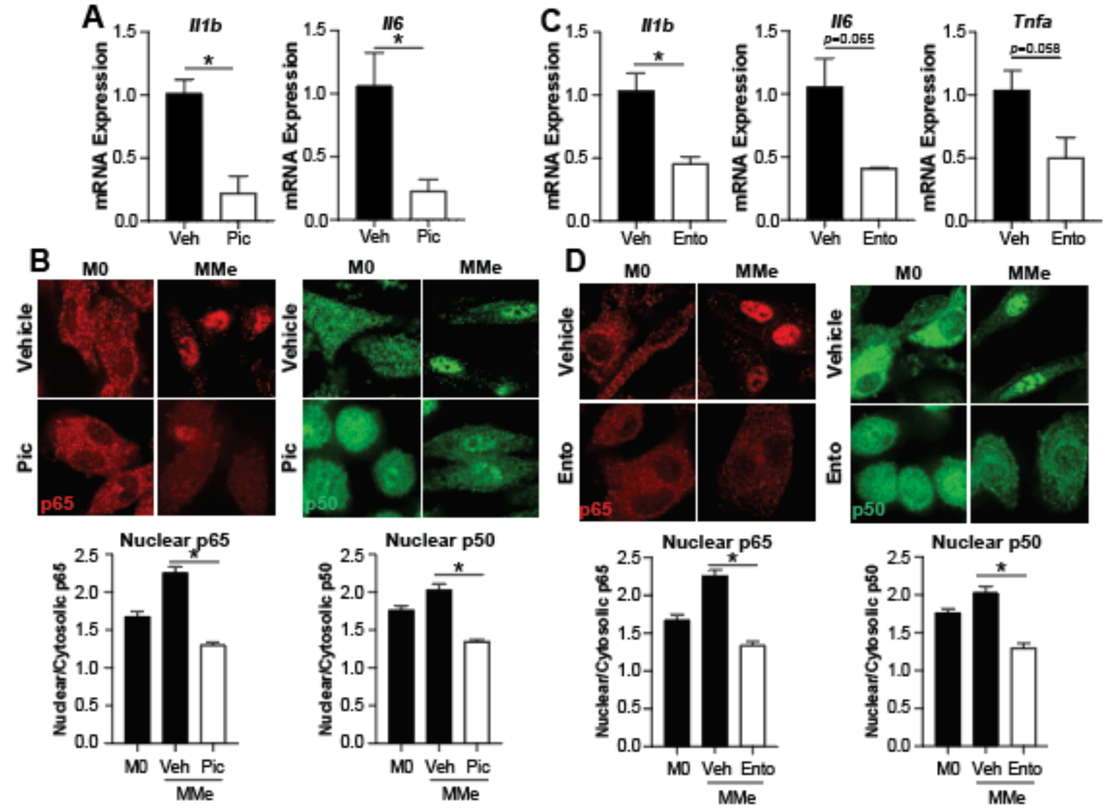


Figure 3.5 SYK contributes to metabolic activation (A) Macrophages were pretreated with vehicle (Veh) or piceatannol (Pic) (25µM, SYK inhibitor) for 1 hour prior to and during MMe activation. mRNA levels are plotted relative to Veh for each group. (C) NFκB p65/p50 nuclear translocation assessed by immunofluroescence. (D) Macrophages pretreated with Veh or entosplenitib (Ento) (25uM, SYK inhibitor). Levels plotted relative to Veh. (D) NFκB p65/p50 nuclear translocation assessed by immunofluroescence. * $p<0.05$ Student's t-test

In B cells, BTK is phosphorylated and activated by spleen tyrosine kinase (SYK)[187]. SYK is a ubiquitous tyrosine kinase essential for both T and B cell receptor signaling. SYK also participates in H_2O_2-induced NFκB activation in Jurkat cells [197]. While our model of BTK activation is based on oxidation, not phosphorylation, this context led us to probe the role of SYK in MMe signaling. To test the importance of SYK in metabolic activation, BMDMs were treated with the SYK inhibitor piceatannol [198]. Expression of *Il1b* and *Il6* were significantly reduced after piceatannol treatment (**Figure 3.5A**) suggesting SYK plays a role in this pathway. Moreover, NFκB nuclear translocation was reduced compared to a vehicle treated control (**Figure 3.5B**). To validate these findings, BMDMs were treated with an alternative FDA-approved SYK inhibitor: entosplenetib [199]. In agreement with the piceatannol treatment, *Il1b* expression was attenuated after entospletinib treatment. A trend towards *Il6* and *Tnfa* reduction was also observed (**Figure 3.5C**) and NFκB nuclear translocation was attenuated (**Figure 3.5D**). Taken together, these data provide evidence that SYK also participates in metabolic activation.

Discussion

Inflammation is mechanistically linked to insulin resistance in mouse models [58,76]. While disrupting this inflammation alleviates insulin resistance in mice, therapies targeting inflammatory cytokines in humans have been largely unsuccessful [200]. These therapies primarily block single inflammatory cytokines, as opposed to mouse studies which can target their cellular source. Thus, developing effect therapies may require a better understanding of the pathways that produce inflammation during obesity. We have shown that ATMs in obesity have a unique metabolically activated phenotype. The signaling events that lead to metabolic activation are largely unknown. Here we probe the signaling pathways that contribute to metabolic activation in an effort to reveal new therapeutic targets.

We demonstrate that MMe uses the transcription factor NFκB. However, NFκB dynamics in MMe are significantly slower and reduced relative to M1. Moreover, MMe NFκB activation is independent of canonical upstream NFκB activators. We also demonstrate that NOX2-derived reactive oxygen species are MMe signaling mediators that activate BTK to propagate the signal through its target PLCγ2. Finally, we show another related tyrosine kinase, SYK, is required for metabolic activation. Taken together, these data suggest that MMe NFκB activation occurs via a pathway independent of classical activation.

NFκB is a transcription factor that contributes to inflammation in both immune and non-immune cells. In macrophages, NFκB is downstream of bacterial detection mechanisms such as TLR2 and TLR4. We demonstrated that NFκB participates in metabolic activation. Unlike classical activation, in which NFκB rapidly moves into the nucleus and peaks at 60 minutes, MMe nuclear NFκB does not increase significantly until 60 minutes and peaks at 2 hours. Additionally, the peak of nuclear NFκB in M1 macrophages is 3 times higher than in MMe macrophages. This higher peak in M1 is consistent with the elevated inflammation in classical activation relative to metabolic activation *in vitro* and *in vivo* [115,201]. This difference in NFκB activation could reflect either a reduced activation of the classical pathway, or the activation of a novel pathway.

In classical activation, IKKβ is phosphorylated and activated before it phosphorylates IκBα to release NFκB and allow it to move to the nucleus. We show that MMe activation requires neither the phosphorylation of IKKβ or degradation of IκBα. The Aggarwal group used IκBα immunoprecipitation and immunoblotting to propose IKKβ independent activation of IκBα in H_2O_2 treated Jurkat cells mediated by tyrosine phosphorylation [197]. Another group showed BTK can directly phosphorylate tyrosine residues on IκBα, bypassing IKKβ and IκBα serine phosphorylation and degradation [202]. Thus we propose that metabolic activation utilizes a similar pathway that circumvents phosphorylation of IKKβ and IκBα and is distinct from classical activation.

This model of metabolic activation begins at the cell membrane when TLR2 binds palmitate and activates NOX2 [78]. We demonstrate that NOX2 propagates metabolic activation by producing reactive oxygen species to function as second messengers. Other groups have shown that cysteine oxidation by H_2O_2 can either activate, as in the case of epidermal growth factor receptor or inhibit enzymatic activity, as in the case of protein-tyrosine phosphatase 1B [203,204]. Our data shows inhibiting BTK attenuated the MMe phenotype, suggesting that the oxidation of BTK has an activating effect.

BTK's tyrosine kinase activity is required for propagating large scale cellular changes such as B cell activation [187]. Our data indicates that BTK is oxidized by NOX2-derived ROS and that its activity contributes to metabolic activation. While initially discovered in B cells, BTK plays a role in macrophage maturation, microbial sensing, phagocytosis, and inflammasome activation[205]. There is no direct evidence of an oxidation event activating BTK in the literature. However, H_2O_2 treatment in B cells has been shown to induce a BTK-dependent activation of PLCγ2 and release of calcium [206].

This activation is notably independent of BTK's SH2 domain, which is important for its interaction with its upstream kinase SYK [207].

SYK is a tyrosine kinase that is critical in lymphocyte activation. It has multiple phosphorylation targets, one of which is BTK. Our data shows that SYK is required for metabolic activation. This was surprising because our model is based on BTK oxidation rather than phosphorylation. The Chock group also showed that both SYK and BTK were required for H_2O_2 activation of B cells. Importantly, they found that eliminating the interaction between BTK and SYK did not abrogate BTK's contribution to H_2O_2 activation [207]. Taken together, this data suggests that BTK and SYK are required for metabolic activation and are either 1) in the same pathway but do not interact or 2) are in divergent pathways.

Here we present a mechanism of metabolic activation dependent on NOX2-derived ROS modification of BTK. BTK then engages it's downstream target PLCγ2

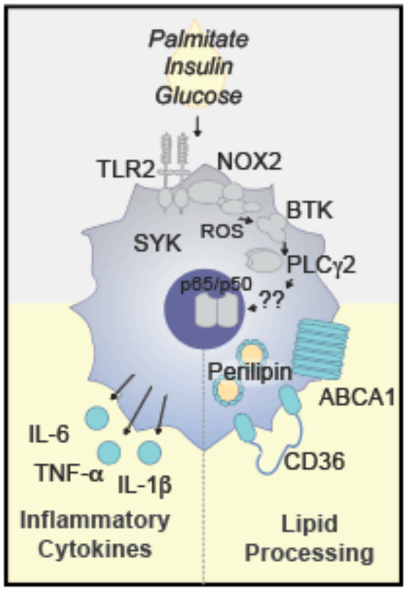


Figure 3.6 Palmitate, insulin, and glucose induce metabolic activation using ROS, BTK, PLCγ2, SYK, and NFκB (p65/p50).

which eventually leads to NFκB p65/p50 movement to the nucleus and inflammatory cytokine production. We also demonstrate that SYK participates in this pathway, though it's role is yet to be defined (**Figure 3.6**). This work provides the foundations of a novel pathway of macrophage activation that reveals potential new therapeutic targets for the treatment of insulin resistance.

CHAPTER 4: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Summary

In mouse models of obesity, macrophages accumulate in adipose tissue and adopt a proinflammatory phenotype that contributes to insulin resistance[51,53]. This mechanistic evidence in mice has made inflammation a therapeutic target for the treatment of type 2 diabetes[200]. However, in humans, both proinflammatory and anti-inflammatory markers on macrophages have been reported in insulin resistance, making the role of ATM inflammation less clear[113,128,152]. Our lab previously reported a novel macrophage phenotype, metabolic activation, that has characteristics of both M1 and M2 macrophages. MMe activation associates with obesity and has been shown to play both protective and pathogenic roles in insulin resistance[78,115]. Therefore, targeting pathogenic MMe activation will require a better understanding of MMe markers in human insulin resistance and of the signaling pathways that gives rise to this phenotype. Here we present a two-fold study on the biology of metabolically activated adipose tissue macrophages. First, we demonstrate a robust relationship between the MMe marker ABCA1 on omental ATMs and insulin resistance in humans. Second, we present a series of tyrosine kinases and alternative activation of NFκB that contribute to metabolic activation *in vitro*, uncovering potential novel therapeutic targets in macrophage activation.

Future Directions

Dissecting the signaling between TLR2 and NOX2 in metabolic activation

We previously demonstrated that TLR2 and NOX2 contribute to metabolic activation *in vitro*[78]. The MMe *in vitro* phenotype is primarily driven by palmitate, a 16 carbon fatty acid that is the major lipid species released in the adipose tissue microenvironment[208]. Palmitate can bind TLR2 and allow it to dimerize with either TLR1 or TLR6. This palmitate-driven dimerization activates the inflammasome to produce IL-1β in THP-1 cells[68]. Knocking out *Tlr2* in mice lowers obesity associated inflammation and insulin resistance in a diet-induced obesity model[75]. We have shown macrophages lacking *Tlr2* cannot become metabolically activated, but still respond to bacterial products[78]. Taken together, this evidence demonstrates MMe activation is initiated by palmitate's engagement of TLR2 and its dimerization partner.

A second protein required for metabolic, but not classical, activation is NOX2[78]. NOX2 is a superoxide producing electron transport chain made up of both membrane-bound and cytosolic subunits that assemble upon activation[182]. Like TLR2, NOX2 is activated in response to palmitate in multiple cell types[209–211]. Mice lacking *Nox2* have attenuated inflammation and improved insulin sensitivity on HFD compared to wildtype controls[78]. Here we've presented evidence that NOX2-derived reactive oxygen species act as second messenger in metabolic activation.

TLR2 and NOX2 activation are some of the initiating events of metabolic activation. Secreted cytokine arrays of *Tlr2* and *Nox2* knockouts after metabolic

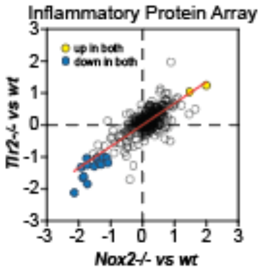


Figure 4.1 *Tlr2* and *Nox2* knockouts have similar effects on cytokine secretion. Mass spectrometry analysis of secreted cytokines from *Tlr2-/-* and *Nox2-/-* BMDMs compared to wildtype

activation show nearly identical alterations relative to MMe activated wildtype BMDMs, suggesting they lie in the same signaling pathway **(Figure 4.1)**. Importantly, TLRs have minimal intracellular domains and transmit a signal into the cell through adaptor proteins[62]. These adaptor proteins and TLRs interact through their respective TIR domains. NOX2 does not contain a TIR domain, making it unlikely to associate directly with TLR2. Thus, while TLR2 and plasma membrane NOX2 clearly perform some of the initiating events of metabolic activation, the mechanism of their interaction is unknown.

NOX2 activation requires the assembly of its cytosolic subunits on the plasma membrane and is controlled at multiple levels by phosphoinositide 3 kinase (PI3K). PI3K produces membrane PIP_3 that provides a binding site for proteins with a pleckstrin homology (PH) or phox homology (PX) domain[212]. In many signaling pathways PIP_3 production attracts protein kinase B (AKT) to the membrane where it's phosphorylated and activated[213]. NOX2 assembly is then initiated when AKT phosphorylates the $p47^{phox}$ subunit of NOX2[214,215]. In MMe activated BMDMs, AKT is phosphorylated early after stimulation **(Figure 4.2).** While this does not prove AKT's involvement, it suggests that AKT is at least activated concurrently with the assembly of NOX2. This assembly is also

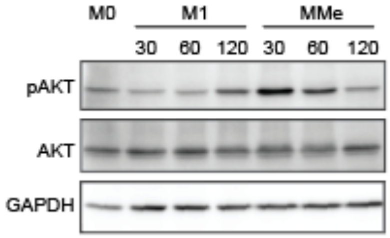


Figure 4.2 Metabolic activation induces early phosphorylation of AKT. Western blot time course of classical and metabolic activation in BMDMs

directly promoted by production of PIP_3, because the cytosolic components of NOX2 contain a PX domain [216]. Based on these data, it is possible that PI3K contributes to metabolic activation by promoting the activation of AKT and assembly of NOX2.

To test this hypothesis, we must develop a system to probe NOX2 assembly and activation. Using immunofluorescence, we would observe movement of proteins such as AKT to the plasma membrane from the cytoplasm. Similar techniques could be used to quantify the assembly of NOX2 by immunofluorescent staining of the cytosolic subunits $p47^{phox}$ and $p67^{phox}$. The phosphorylation and activation of the $p47^{phox}$ subunit would be assessed by immunoblotting. These systems will be utilized in conjunction with PI3K and AKT inhibitors to assess their contribution to both NOX2 assembly and metabolic activation as a whole.

Interrogate the role of SYK in metabolic activation

We found the tyrosine kinase SYK is required for metabolic activation. SYK plays a critical role in the activation of leukocytes by recognizing and binding to phosphorylated ITAM regions of the BCR and TCR with its SH2 domain[217]. More recently, SYK has been shown to participate in Fc-receptor signaling in multiple hematopoietic cells lines[218]. Whole body knockouts of SYK are embryonic lethal, but chemical inhibition of SYK results in generalized immunosuppression[219]. SYK exists in an autoinhibited state in the cytosol. This autoinhibition is relieved when SYK's SH2 domain binds phosphorylated tyrosines. Once active, SYK is able to auto phosphorylate and dissociate from the phosphorylated tyrosines while remaining active[217].

In B cell signaling, activated SYK phosphorylates and activates BTK at tyrosine[551]. Importantly, SYK does not directly bind to BTK. Rather, their association is mediated by the protein BLNK, which binds BTK's SH2 domain and brings the two proteins into proximity. Specific deletion of BTKs SH2 domain interrupts this interaction in B cells and prevents SYK phosphorylation of BTK and activation of B cells[220]. Importantly, our model of BTK activation is based on cysteine oxidation rather than tyrosine phosphorylation. There is existing evidence that H_2O_2-induced activation of BTK does not require the interaction of SYK and BTK. Specific deletion of BTK's SH2 domain in DT40 cells, which interrupts its association with BLNK and SYK, did not impair BTK's ability to activate PLCγ2 in response to H_2O_2[206,207]. This presents the possibility that, while both SYK and BTK are required for metabolic activation, they do no interact in the pathway. If SYK is not phosphorylating BTK, this raises the question of SYK's role in metabolic activation.

A potential role for SYK in metabolic activation is in the portion of the pathway between TLR2 and NOX2. TLRs interact with the intracellular space through adaptor proteins that contain a TIR domain[62]. TLR2 specifically uses a combination of MAL/TIRAP, which directly binds the TLR, and MyD88 which binds MAL/TIRAP[221]. In canonical TLR2 signaling, MyD88 recruits a series of kinases that result in NFκB activation. MyD88 also contains a hemi-ITAM motif that SYK is able to bind and initiate its activation[222,223]. SYK is also able to phosphorylate and activate a host of targets, one of which is PI3K, leading to the generation of PIP_3 and recruitment of PH/PX domain containing proteins to the plasma membrane[212,224]. Based on the literature, and the lack requirement of BTK phosphorylation in our model, we hypothesize that SYK contributes to the activation of PI3K and the recruitment of PH/PX-domain containing proteins to the plasma membrane.

To test this hypothesis would use the same system of monitoring AKT movement to the plasma membrane with immunofluorescence. Importantly, insulin is added along with palmitate in MMe activation *in vitro*. The insulin receptor also activates PI3K and it will be important to distinguish the distinct contributions of SYK and the insulin receptor.

Identify the mechanism of IκBα-degradation independent NFκB activation

We have shown NFκB contributes to metabolic activation *in vitro*. Specifically, the p65 and p50 subunits of NFκB translocate to the nucleus after one hour of MMe activation and that translocation is required for expression of inflammatory cytokines. The p65 and p50 subunits are also required for classical activation but move to the nucleus within 15 minutes of M1 activation. Before activation, NFκB is sequestered in the cytosol by IκBα, which covers NFκB's nuclear localization sequence. During classical activation, IκBα is phosphorylated at $Ser^{32/36}$ by IKKβ and degraded, thus allowing NFκB to move to the nucleus and initiate transcription[79]. We have demonstrated that NFκB nuclear translocation in MMe activation does not require IKKβ activation or IκBα degradation, but the mechanism of IκBα release of NFκB in metabolic activation is unknown.

Other groups have shown NFκB activation independent of IκBα degradation. In Jurkat cells H_2O_2 can induce *Tnfa* expression without IκBα degradation[197]. In this system immunoprecipitation was used to show a generalized increase in tyrosine phosphorylation of IκBα. Other studies confirm the tyrosine phosphorylation of IκBα in response to reoxygenation or epidermal growth factor receptor signaling and implicate tyrosine 42 [225–228]. Alternatively, a more recent study suggests that BTK can directly bind IκBα and phosphorylate it a tyrosine[289] and tyrosine[305202]. Based on this context, we posit that tyrosine phosphorylation allows IκBα to dissociate from NFκB in metabolic activation.

To test this, we will first need to identify binding partners and modification of IκBα using co-immunoprecipitation and mass spectrometry. After identifying a phosphorylation site, CRISPR/Cas9-mediated targeted mutation of that site will allow us to assess its contribution to metabolic activation. Identification of a novel kinase-IκBα interaction may provide the most straightforward therapeutic target to inhibit MMe activation. Both of the kinases we've identified in metabolic activation, SYK and BTK, are required for the development and activation of leukocytes. While approved drugs exists to target both of these kinases, they present the risk of impaired leukocyte development and broad immunosuppression. Identification of the IκBα modification and responsible kinase may provide a promising new therapeutic target that is more specific to macrophages and metabolic activation.

Targeting tyrosine kinases in macrophages to disrupt metabolic inflammation

The identification of SYK and BTK's participation in metabolic activation were performed *in vitro*. To test the contribution of SYK and BTK to metabolic activation *in vivo* we will acquire myeloid-specific *Syk* and *Btk* knockout mice. First, these mice will be used to make BMDMs to validate the results of our inhibitor experiments.

Importantly, the combination of knockout BMDMs and inhibitors will be critical for ruling out unintended effects of the inhibitors. To test the contribution of SYK and BTK *in vivo* we will general LysM-Cre driven myeloid specific knockouts of *Syk* and *Btk*. Eight week-old male mice will be placed on a 60% HFD for 12 weeks. Insulin resistance will be measured at 6 weeks HFD and 12 weeks HFD using intraperitoneal insulin and glucose tolerance tests as well as fasting glucose and insulin measurements. Local adipose tissue insulin resistance will be quantified using AKT phosphorylation in an *ex vivo* adipose insulin resistance assay. To quantify metabolic activation we will measure adipose tissue inflammation using qPCR and flow cytometry on isolated ATMs. Adipose tissue health will be monitored by quantifying adipocyte size and crown-like structure density using immunofluorescence.

Notably, inhibitors of both SYK and BTK are approved or in the process of approval by the Food and Drug Administration. Entospletinib, a competitive inhibitor that interrupts SYK's kinase function, is currently in phase 3 trials for treatment of chronic lymphocytic leukemia[229]. Second, ibrutinib is an inhibitor that binds to the allosteric inhibitor region of BTK and is approved for the treatment of mantle cell leukemia, chronic lymphocytic leukemia, and Waldenstrom Macroglobulinemia[230]. Chemical inhibition of SYK and BTK have shown some efficacy in relieving insulin resistance in mouse models[193,231]. While we have shown these drugs attenuate MMe activation in BMDMs, their ability to do so in ATMs *in vivo* and its effect on insulin resistance remain unknown. While these drugs have the potential to alleviate inflammation in ATMs, they have a profound ability to decimate the leukocyte population. To test their efficacy *in vivo* we must first be able to target them specifically to macrophages. Our lab is

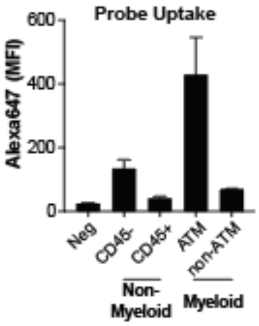


Figure 4.3 dsDNA probe is primarily taken up in adipose tissue macrophages. Mean fluorescence intensity of Alexa647-conjugated probe in non-immune SVC (CD45-), non-myeloid immune cells (CD45+CD11b-) ATMs (CD45+CD11b+F4/80+), and non-ATM myeloid cells (CD45+ CD11b+ F4/80-).

developing a DNA-based system that can target inhibitors to macrophages. Double-stranded DNA (dsDNA) contains a negatively charged backbone making it an ideal ligand for scavenger receptors which are known to bind to various polyanionic ligands and present abundantly on macrophages. Using a small (0.04kb) dsDNA as a backbone we target various inhibitors to the macrophage endosomal system. Here we show the specific targeting of Alexa 647 labelled dsDNA to ATMs after IP injection (**Figure 4.3**).

We would first assess the ability of our dsDNA structure to target SYK and BTK inhibitors to macrophages and inhibit metabolic activation. We would then challenge C57BL/6 mice with a 60% HFD and treat them with our dsDNA-inhibitor conjugates. Male mice will be placed on a 60% HFD for 6 weeks before being treated with dsDNA-conjugated entospletinib (100mg/kg) or ibrutinib (30mg/kg) 5 times a week for an additional 6 weeks while on HFD[193,232]. The mice will be metabolically phenotyped and their ATM metabolic activation will be quantified as described above.

This work has informed our understanding of the role of MMe ATMs during obesity and insulin resistance. While still preliminary, the data presented here addresses the question of the function of ATMs in metabolic disease at two levels. At the clinical level we provide a novel non-inflammatory ATM marker associated with insulin resistance. *In vitro*, we are closer to understanding how macrophages respond to fatty acids and how those pathways can be perturbed for the benefit of those suffering from metabolic disease.

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