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Role of MAP4K4 Signaling in Adipocyte and Macrophage Derived Inflammation: A Dissertation

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THE ROLE OF MAP4K4 SIGNALING IN ADIPOCYTE AND MACROPHAGE DERIVED INFLAMMATION

A Dissertation Presented

By

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Abstract

Human obesity is increasing globally at an impressive rate. The rise in obesity has led to an increase in diseases associated with obesity, such as type 2 diabetes. A major prerequisite for this disease is the development of insulin resistance in the muscle and adipose tissues. Interestingly, experiments in rodent models suggest that adipocytes and macrophages can profoundly influence the development of insulin resistance. Accordingly, the number of adipose tissue macrophages increases substantially during the development of obesity. Numerous research models have demonstrated that macrophages promote insulin resistance by secreting cytokines, like TNFα, which impair whole body insulin sensitivity and adipose tissue function. Additionally, enhancements of murine adipose function, particularly glucose disposal, prevent the development of insulin resistance in mice on a high fat diet. Thus, mechanisms which enhance adipose function or attenuate macrophage inflammation are of interest.

Our lab previously identified mitogen activated protein kinase kinase kinase kinase 4 (MAP4K4) as a potent negative regulator of adipocyte function. In these studies, TNFα treatment increased the expression of adipocyte MAP4K4. Furthermore, the use of small interfering RNAs (siRNA) to block the increase in MAP4K4 expression protected adipocytes from some of the adverse effects of TNFα. Because MAP4K4 is a potent negative regulator of adipocyte function, an understanding of the mechanisms by which TNFα regulates MAP4K4 expression is of interest. Thus, for the first part of this thesis, I characterized the signaling pathways utilized by TNFα to regulate MAP4K4

expression in cultured adipocytes. Here I show that TNFα increases MAP4K4 expression through a pathway requiring the transcription factors activating transcription factor 2 (ATF2) and the JUN oncogene (cJUN). Through TNFα receptor 1 (TNFR1), but not TNFR2, TNFα increases MAP4K4 expression. This increase is highly specific to TNFα, as the inflammatory agents IL-1β, IL-6 and LPS did not affect MAP4K4 expression. In agreement, the activation of cJUN and ATF2 by TNFα is sustained over a longer period of time than by IL-1β in adipocytes. Finally, MAP4K4 is unique as the expression of other MAP kinases tested fails to change substantially with TNFα treatment.

For the second part of this thesis, I assessed the role of MAP4K4 in macrophage inflammation *in vitro* and *in vivo*. To accomplish this task, pure β1,3-D-glucan shells were used to encapsulate siRNA. Glucan shells were utilized because they are effectively taken up by macrophages which express the dectin-1 receptor and they survive oral delivery. I demonstrate that these β1,3-D-glucan encapsulated RNAi particles (GeRPs) are efficiently phagocytosed and capable of mediating the silencing of multiple macrophage genes *in vitro* and *in vivo*. Importantly, oral treatment of mice with GeRPs fails to increase plasma IFNγ and TNFα or alter serum AST and ALT levels. Orally administered GeRPs are found in macrophages isolated from the spleen, liver, lung and peritoneal cavity and mediate macrophage gene silencing in these tissues.

Utilizing this technology, I reveal that MAP4K4 augments the expression of TNFα in macrophages following LPS treatment. Oral delivery of MAP4K4 siRNA in GeRPs silences MAP4K4 expression by 70% and reduces basal TNFα and IL-1β expression

significantly. The depletion of MAP4K4 in macrophages protects 40% of mice from death in the LPS/D- galactosamine (D-GalN) model of septicemia, compared to less than 10% in the control groups. This protection associates with significant decreases in serum TNFα concentrations following LPS/D-GalN challenge. Consistent with reduced macrophage inflammation, hepatocytes from mice treated orally with GeRPs targeting MAP4K4 present less apoptosis following LPS/D-GalN treatment. Thus, MAP4K4 is an important regulator of macrophage TNFα production in response to LPS.

The results presented here add to the knowledge of MAP4K4 action in adipocyte and macrophage inflammation substantially. Prior to these studies, the mechanism by which TNFα controlled MAP4K4 expression in adipocytes remained unknown. Considering that MAP4K4 is a negative regulator of adipocyte function, identifying the mechanisms that control MAP4K4 expression was of interest. Furthermore, the role of macrophage MAP4K4 in LPS stimulated TNFα production was also unknown. To address this question *in* vivo, new technology specifically targeting macrophages was needed. Thus, we developed a technology for non toxic and highly specific macrophage gene silencing *in vivo*. Considering that macrophages mediate numerous diseases, the application of GeRPs to these disease models is an exciting new possibility.

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List of Frequently Used Abbreviations

Abbreviation	Term
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
TNFα	Tumor necrosis factor alpha
siRNA	small interfering RNA
RNAi	RNA interference
GeRPs	β1,3-D-glucan encapsulated RNAi particles
cJUN	JUN oncogene
ATF2	activating transcription factor 2
TNFR1	TNFα receptor 1
TNFR2	TNFα receptor 2
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
IFNγ	Interferon γ
LPS	Lipopolysaccharide
D-GalN	D-galactosamine
IR	Insulin receptor
IRS-1/2	Insulin receptor substrate 1/2
PI3K	phosphatidylinositol 3-kinase
AKT	protein kinase B
ALT	alanine aminotransferase
AST	aspartate aminotransferase
MAPK	Mitogen activated protein kinase
PPARγ	peroxisome proliferator-activated receptor gamma

C/EBPα	CCAAT/enhancer binding protein alpha
FFAs	free fatty acids
ERK1/2	extracellular-signal regulated kinases 1 and 2
JNK1/2	cJUN n-terminal Kinase 1 and 2
MKK4/7	MAPK Kinases 4 and 7
p38 SAPK	the p38 stress activated protein kinase
IKKβ	the inhibitory κ B Kinase β
IκBα	inhibitor-κ B protein α
NFκB	nuclear factor κ B
AP-1	activator protein 1
TLR4	Toll-like receptor 4

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CHAPTER I: Introduction

Normally following an increase in blood glucose levels, pancreatic beta cells release copious amounts of insulin into the blood to lower blood sugar levels. This function of insulin is mediated primarily on the 3 major insulin target tissues, the liver, adipose and muscle. In the liver, insulin functions to suppress hepatic glucose production by inhibiting glycogenolysis and gluconeogenesis while promoting the storage of glucose as glycogen. In muscle and adipose tissue, insulin promotes the facilitated uptake of glucose and the conversion of the sugar to glycogen and triglycerides respectively. The failure of insulin to elicit a proper response on these target tissues results in disruption of normal blood sugar levels.

Until recently, very little regarding the cellular mechanisms of insulin action on the target tissues was known. For more than 50 years after the discovery of insulin by Banting and Best, the majority of studies focused on the *in vivo* effects of insulin on the whole body. Little knowledge existed on the mechanisms of insulin action on targeted cells. However, all this changed upon the discovery of the insulin receptor[1,2]. Soon investigators realized that the receptor possessed a tyrosine kinase activity[3-5]. Since then, numerous insights into the intracellular actions of insulin have been made, yet much remains to be discovered.

Insulin Signaling

The insulin receptor (IR) comprises two extracellular α subunits, and two transmembrane β subunits. Binding of insulin stimulates receptor auto-phosphorylation

through the protein tyrosine kinase activity located on the β subunits. The auto-phosphorylation event occurs as one β subunit trans-phosphorylates the other. Auto-phosphorylation increases the receptor's catalytic activity over 200 fold[6], and allows for the recruitment and phosphorylation of adaptor proteins[7]. The first adaptor protein identified was the insulin receptor substrate-1 (IRS-1), which is recruited through it's pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains[8]. Other adaptor proteins interact with the insulin receptor, including IRS-2, IRS-3 and IRS-4 along with SHC, GAB2, DOCK1/2 , APS and others[9-12]. However, work with transgenic mice suggests that the majority of insulin's metabolic effects are mediated by mainly IRS-1 and IRS-2[13,14]. Docking of the IRS proteins allows for their tyrosine phosphorylation by the IR and the subsequent formation of binding sites for Src-homology-2 (SH2) domain containing proteins. For Insulin action, the most relevant IRS binding proteins are the SH2 domain containing regulatory subunits of phosphatidylinositol 3-kinase (PI3K) of the class 1A family and the growth factor receptor bound protein 2 (GRB2)[7]. These two adaptor molecules regulate the two main conduits of insulin action, PI3K and the serine/threonine protein kinase B (AKT) and GRB2 and the mitogen activated protein kinases (MAPK). AKT regulates glucose utilization, production and uptake while MAPK regulate cell growth, gene expression and differentiation. The effects of these pathways are summarized below and in Figure 1.1.

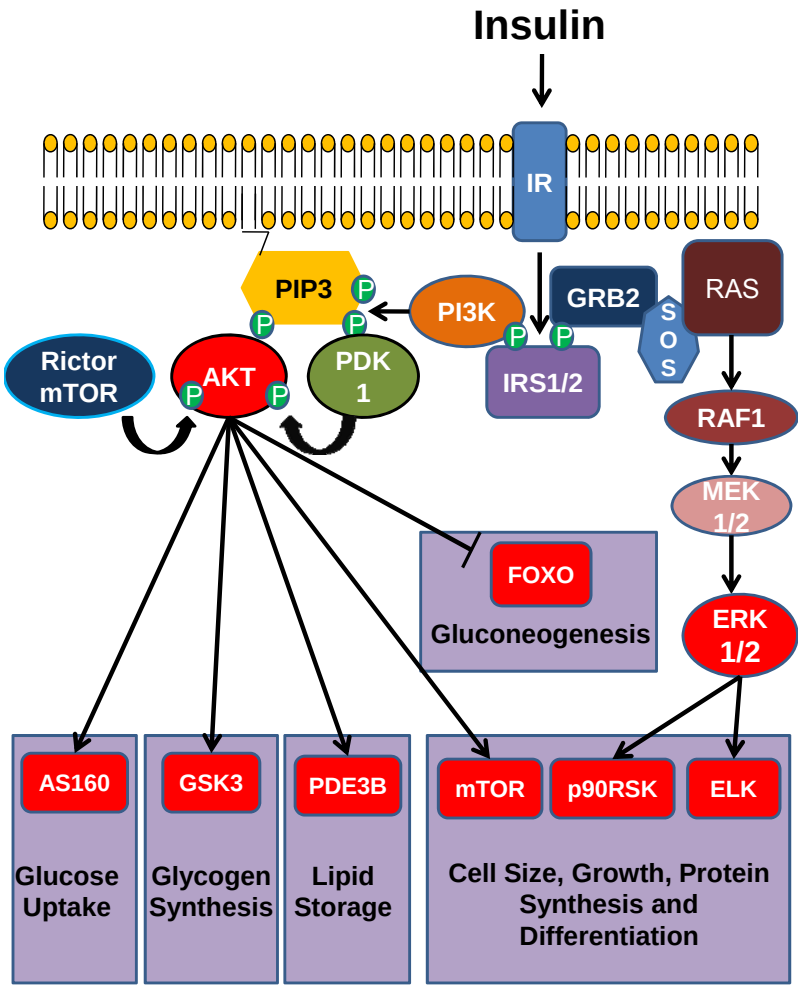

Figure 1.1 The metabolic pathways regulated by insulin action. Insulin binding the insulin receptor (IR) activates the receptor's tyrosine kinase activity and stimulates the tyrosine phosphorylation of the insulin receptor substrate adaptor protein 1 (IRS1). Tyrosine phosphorylated IRS1 serves as a docking substrate for phosphatidylinositol 3-kinase (PI3K) and the growth factor receptor bound protein 2 (GRB2) to activate to activate the protein kinase B (AKT) and extracellular-signal regulated kinase (ERK) branch of insulin mediated metabolic signaling. Activation of AKT and ERK results in the regulation of the targets and processes indicated in the diagram. Some of these processes are cell type specific, such as the stimulation of glycogen synthesis in muscle tissue, triglyceride synthesis in adipose tissue, glucose uptake in adipose and muscle tissue and the inhibition of gluconeogenesis in liver tissue.

The PI3K/AKT Branch of Insulin Signaling

The functional PI3K enzyme that mediates insulin action consists of both a regulatory and catalytic subunit of Class 1A. Class 1A PI3K kinases consist of 3 known catalytic subunits and 8 regulatory subunits generated from the alternative splicing of 3 genes, of which multiple isoforms of both subunits are expressed in the insulin sensitive tissues[15]. The p85α regulatory and the p110β catalytic subunit are the subunits to which insulin's metabolic effects have been attributed[7]. The importance of PI3K is highlighted by the fact that dominant negative PI3K subunits and the PI3K inhibitors LY294007 and wortmanin virtually abolish all of the hormone's effects[16].

As mentioned earlier, insulin stimulated tyrosine phosphorylation of the IRS proteins increases the binding of the PI3K regulatory subunits through two SH2 domains. This interaction is necessary for the full activation of the catalytic subunit [17]. Activated PI3K enzyme phosphorylates phosphatidylinositol (PI) molecules on the 3 position of the inositol ring. Particularly important is the phosphorylation of PI (4,5)-bisphosphate on the 3 position, forming PI (3,4,5)-triphosphate, an important membrane docking phospholipid for PH domain containing proteins. For Insulin action, the two most critical PIP3 binding proteins are the 3-phosphoinositide-dependent protein kinase 1 (PDK1), and AKT. Interestingly, PDK1 exists in a constitutively active state that is not altered by growth factor or hormonal signaling [18,19]. Instead, generation of PIP3 by PI3K changes the cytosolic localization of PDK1 to the plasma membrane where PDK1 readily phosphorylates the activation loop of AKT on T308[20]. However, T308 phosphorylation must be accompanied by phosphorylation of AKT on S473 for full AKT activation. Until recently, the identity of this putative PDK2 has remained a mystery.

New data suggests that the rapamycin-insensitive companion of mTOR (RICTOR)-mammalian target of rapamycin (mTOR) complex may function as PDK2 since genetic ablation of RICTOR in mouse muscle impairs the insulin mediated S473 phosphorylation[21,22].

Upon activation, AKT phosphorylates numerous substrates to control the metabolic actions of insulin[7]. These include including Rab GTPase activating protein (GAP) with a molecular weight of 160 kDal (AS160), the forkhead box o (FOXO) transcription factors, glycogen synthase kinase-3 (GSK3), tuberos sclerosis complex 2 (TSC2), phosphodiesterase 3B (PDE3B) and other proteins to control the metabolic actions of insulin[7].

Regulation of Glucose Uptake

Perhaps the ability of insulin to lower blood sugar levels is the hormone's most commonly recognized function. Part of this effect is achieved through the facilitated uptake of glucose by the insulin sensitive glucose transporter, isoform 4 (GLUT4), mainly in muscle tissue[23]. The importance of GLUT4 in regulating whole body glucose homeostasis is highlighted by numerous genetic mouse models. Heterozygous, GLUT4 +/- mice show decreased insulin sensitivity, and a predisposition towards diabetes[24]. However, this defect can be overcome simply through muscle specific GLUT4 over-expression[25]. Furthermore, in normal mice, adipose and muscle tissue specific over-expression of GLUT4 protects from diet induced diabetes[26,27]. Conversely, specific depletion of GLUT4 in mouse adipose or muscle tissues results in glucose intolerance and diabetes[28,29]. Despite the success of rodent studies in demonstrating the

importance of GLUT4 in maintaining glucose homeostasis, studies in cellular models were needed to determine the mechanistic effects of insulin on GLUT4.

Interestingly, insulin regulates GLUT4 plasma membrane translocation through a PI3K/AKT signaling mechanism. Pharmacological and dominant negative inhibitors of PI3K have demonstrated the requirement of PIP3 for GLUT4 translocation[30-32]. Thus, the targets downstream of AKT controlling GLUT4 translocation remain an area of intense investigation. Recent work has recognized the RAB GAP, AS160, as a downstream target of AKT that regulates GLUT4 translocation[33]. Members of the Ras superfamily of monomeric G-proteins (RAB) are the functional regulators of intracellular vesicle trafficking[34]. Studies suggest that AS160 may retain GLUT4 in an intracellular compartment until insulin activates AKT. Active AKT then phosphorylates and inactivates AS160. Supporting this model, AS160 depletion by RNAi resulted in higher basal levels of GLUT4 on the adipocyte plasma membrane[35,36]. However, other AKT substrates that regulate GLUT4 retention must exist as knockdown of AS160 only released part of the GLUT4 intracellular pool[35,37].

Regulation of Glycogen Synthesis by Insulin

Upon uptake, glucose is converted to glycogen in muscle and liver by the enzyme glycogen synthase (GS). GS is activated by glucose-6-phosphate and also by insulin within minutes of the hormone binding it's receptor[38]. Activation of GS was the first demonstration of a metabolic enzyme being affected by insulin[39,40]. The conversion of glucose to glucose-6-phosphate by hexokinase (muscle) and glucokinase (liver) occurs simply by the cellular uptake of glucose by cells. However, upon activation by insulin, GS transforms from a highly phosphorylated, low activity form, to a poorly

phosphorylated, but highly active form. Interestingly, the low phosphorylated isoform is less dependent on glucose-6-phosphate for activity[41]. Insulin activates GS through the phosphorylation of glycogen synthase kinase 3 (GSK3) by AKT in muscle[42]. The AKT phosphorylation inhibits GSK3 activity, causing a decrease in the phosphorylation state of GS and a subsequent increase in muscle glycogen synthesis following insulin stimulation. Interestingly, phosphorylation of GSK3 by AKT is not the major mechanism driving GS activation in liver. Instead, dephosphorylation of GS stimulates liver glycogen production[43].

The dysregulation of GS may be an important feature in type 2 diabetes as diabetic humans have a dramatic suppression of hepatic glycogen synthesis. Potent inhibitors of GSK3 reduce rodent blood glucose levels, primarily by increasing hepatic glycogen synthesis and decreasing gluconeogensis[44]. Thus, enhancing GS activity remains a potential therapeutic target for improving glucose metabolism.

FOXO Regulation of Metabolic Gene Transcription

The FOXO transcription factor family regulates genes critical to metabolism in all metazoans. In addition to metabolic gene regulation, the 4 mammalian homologues, designated FOXO1, FOXO3a, FOXO4 and FOXO6, also regulate other diverse processes, including cell cycle, apoptosis, DNA repair and oxidative stress[45,46]. Under basal conditions, the FOXO transcription factors actively promote the transcription of target genes. However, upon insulin stimulation, AKT phosphorylates the FOXO proteins, leading to their rapid exclusion from the nucleus and inactivation[47].

FOXO regulation by AKT controls the expression of gluconeogenic and lipogenic gene expression during fed and fasted periods. Although, all metabolic tissues require the regulation of FOXO for normal functioning, FOXO regulation by AKT in the liver is also important in controlling blood glucose levels. Hepatocytes upregulate gluconeogenesis by increasing the expression of essential gluconeogenic enzymes, such as glucose-6-phosphatase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carbokinase[48]. The increase in expression of these enzymes is achieved in part by FOXO1 during periods of reduced insulin/AKT signaling. In agreement, deficiency of the FOXO1 gene in mice improves insulin sensitivity through a reduction of hepatic glucose output caused by the reduced expression of gluconeogenic genes[49,50]. Conversely, expression of constitutively active FOXO1 in hepatocytes increases gluconeogenic gene expression, elevates blood glucose and insulin levels, resulting in a failure to maintain euglycaemic in mice[51].

In the adipose and muscle tissues, FOXO transcription also promotes a metabolic switch during periods of fasting. In adipose tissue, FOXO1 suppresses the promoter of peroxisome proliferator-activated receptor gamma (PPARγ) and hinders PPARγ target gene transcription[52,53]. In muscle, the FOXO proteins regulate the switch from carbohydrate fuel sources to fatty acids during fasting[54,55]. Altogether, dysregulation of FOXO in any of these tissues, due to decreased insulin signaling, can potentially have drastic effects on metabolic gene expression.

TSC2 and mTOR Nutrient Sensing and Control of Cell Growth

The ability to sense the nutritional status and thus the proper time for cell growth is vital for cell survival. The sensing of nutritional state is partly mediated by insulin

through the phosphorylation of TSC2 by AKT[56-58]. This phosphorylation event is required for the insulin growth signal, as mutation of the phosphorylation sites blocked the ability of insulin to stimulate cell growth[57,58]. Under basal conditions, the GAP domain of TSC2 promotes the hydrolysis of GTP by RHEB, a member of the Ras super family[59]. In this manner, TSC2 exists in a complex with TSC1, RHEB and mTOR suppressing mTOR activity. However, upon AKT phosphorylation, the TSC complex is inactivated allowing for the activation of mTOR.

Downstream of mTOR, two targets are crucial for the regulation of cell growth and size. The first target, elongation initiation factor 4E binding protein (eIF4E-BP), inhibits translation by binding eIF4E. Active mTOR phosphorylates several sites on eIF4E-BP, thus relieving the inhibition of protein translation[60]. The second target important in cell growth is the p70 ribosomal protein S6 kinase (p70S6K). The phosphorylation of p70S6K by mTOR increases the activity and subsequent phosphorylation of the downstream ribosomal protein target S6. Interestingly, the phosphorylation of S6 does not affect translation but instead seems to determine cell size as mutation of the mTOR phosphorylation sites failed to elicit any changes in translation [61]. Furthermore, in this report, the authors demonstrate that rapamycin treatment failed to further decrease cell size in the S6 mutant cells, implying that S6 regulates cell size, but not translation.

Insulin Regulation of Lipolysis through PDE3B

The release of free fatty acids (FFAs) by adipose tissue is an important metabolic parameter regulated by PI3K/AKT signaling. For example, following an overnight fast, more than 70% of the total body expenditure can be accounted for by lipid oxidation[62].

It is during periods of fasting that catecholamines stimulate adrenoreceptors which in turn activate adenyl cyclase and increase intracellular cAMP levels. The increase in cAMP activates the cAMP regulated protein kinase A (PKA) which phosphorylates and stimulates hormone sensitive lipase (HSL). Active HSL catalyzes the conversion of stored triglycerides to glycerol and FFAs[63]. However, the production of FFAs by adipose tissue is not required during the fed state. Furthermore, FFA production must be tightly regulated as an excess of FFAs has been associated with insulin signaling and metabolic defects that will be discussed later in this chapter[64]. To balance FFA release, insulin activation of PI3K promotes the AKT induced phosphorylation of PDE3B during the fed state[65]. Phosphorylated PDE3B hydrolyzes cAMP and thus reduces intracellular cAMP levels and HSL activity. The net result is a switch from triglyceride hydrolysis to triglyceride synthesis during periods of nutritional excess. Disruption of this signaling cascade is a major contributor to the elevated levels of FFAs observed in diabetic patients[63].

Other AKT Targets

The AKT targets discussed above are of immense importance to metabolic disease. However, other targets do exist whose importance may be less defined for metabolic disease. These include the phosphorylation and subsequent inactivation of the pro-apoptotic BCL2 protein BAD, the phosphorylation of endothelial nitric oxide synthase which is thought to increase its activity and promote healthy vascular endothelium, and the phosphorylation of p21cip to promote cell cycle progression[66-68]. Undoubtedly, more targets of AKT exist that may have an even more profound impact

on metabolism than the targets discussed above. Future work will no doubt highlight the importance of these new targets.

Insulin Activation of Mitogenic Gene Expression

GRB2, an SH2 motif containing adaptor protein, is recruited to the IRS proteins following insulin induced tyrosine phosphorylation[69]. GRB2 constitutively associates with the guanine nucleotide-exchange factor son of sevenless (SOS), which catalyzes the exchange of GDP for GTP in the membrane bound GTPase RAS[70]. GTP bound RAS recruits and activates the kinase (v-raf-leukemia viral oncogene 1) RAF1. Activated RAF1 starts a serine and threonine phosphorylation cascade that begins with the phosphorylation and subsequent activation of mitogen activated protein kinase kinases 1 and 2 (MEK1/2). Activated, MEK1/2 then phosphorylates and activates the extracellular-signal regulated kinases 1 and 2 (ERK1/2)[71]. ERK1/2 in turn phosphorylates numerous substrates, including the ternary complex factor ETS family members like ELK1. These transcription factors complex with serum response factors and promote the expression of genes containing serum response elements. Typically, these gene products promote cellular growth and proliferation[72,73]. Additionally, activation of ERK1/2 promotes cellular growth and proliferation through activation of p90 ribosomal S6K (p90S6K). Activated p90S6K stimulates protein synthesis through the phosphorylation of the ribosomal S6 subunit. The mitogenic effects of insulin on ERK1/2 are conserved among numerous growth receptor signals in virtually all cells[73]. Thus, through the activation of the ERK signaling pathway, insulin further promotes cell growth, proliferation and differentiation.

Insulin Resistance

Insulin resistance is the failure of insulin to modulate the signaling pathways discussed above. In adipose tissue, insulin resistance results in decreased triglyceride synthesis in response to insulin and increased FFA release. In muscle tissue, the primary defects are reduced insulin stimulated glucose uptake and storage as glycogen. Insulin resistance also fails to switch hepatocytes from glucose production to glycogen synthesis. The net result is an increase in serum FFAs and the dysregulation of blood glucose levels. If left unchecked, insulin resistance progresses towards type 2 diabetes.

Numerous agents and conditions cause insulin resistance in both humans and in rodent models. These include septicemia, acidosis, chronic inflammation, elevated FFAs, obesity and drugs such as glucocorticoids[74-77]. However, most of these causes are unique clinical conditions that fail to represent the majority of instances of insulin resistance. In fact, obesity correlates the most frequently with insulin resistance[63]. Interestingly, obesity associates with other factors that cause insulin resistance, such as elevated serum, muscle and liver tissue FFA levels, and chronic low grade inflammation. Although the exact basis of insulin resistance in obesity remains only partly understood, much work has elucidated some common themes that can be applied to humans. The pathways discussed below and summarized in Figure 1.2 contribute significantly to the development of insulin resistance.

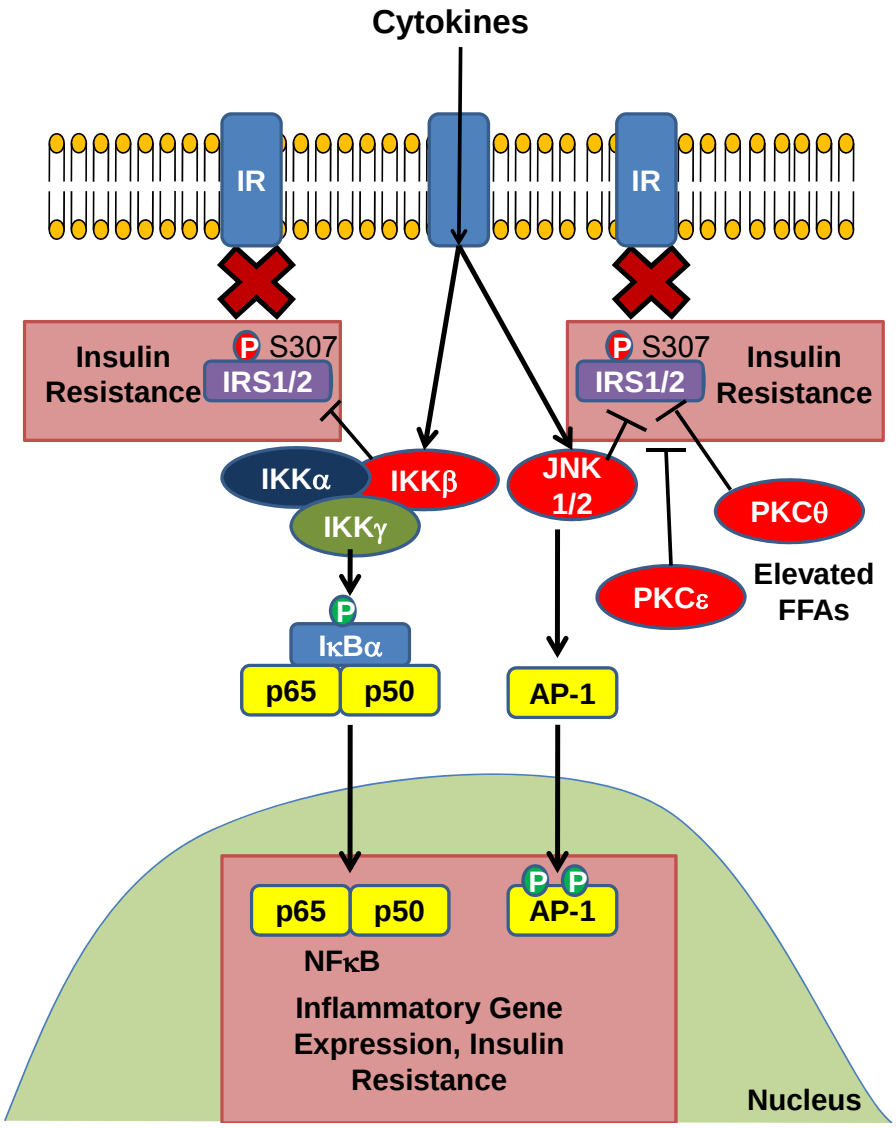

Figure 1.2 Intracellular signaling pathways contributing to inflammation and insulin resistance. Two major pathways for insulin resistance are the activation of cJUN N-terminal Kinases 1/2 (JNK1/2) and IKKβ and NFκB transcription. Both of these pathways regulate cytokine expression and inflammation in macrophages and adipocytes. While the primary role of JNK1/2 is thought to be the phosphorylation of IRS1/2, new data suggests that deletion of JNK1 in hematopoietic cell types influences cytokine expression. Additionally, IKKβ mediated gene transcription causes localized hepatic insulin resistance. The protein kinase C theta (PKCθ) and episilon (PKCε) isoforms contribute to free fatty acid (FFA) induced insulin resistance in muscle and liver respectively, by phosphorylating IRS1 on serine 307 which inhibits IRS1 association with the IR. Both JNK and IKKβ are also capable of phosphorylating S307.

Serine Phosphorylation of IRS proteins

Serine phosphorylation of the IRS proteins is a major contribution to insulin resistance. Numerous protein kinases, to be discussed in the upcoming text, phosphorylate IRS-1 on serine residues. These include, the cJUN n-terminal Kinase (JNK)[78], the inhibitory κ B Kinase β (IKKβ)[79], protein kinase C θ (PKCθ)[80], GSK3, mammalian target of rapamycin complex‑1 (mTORC1) and p70S6K[81], which all phosphorylate various serine residues of the IRS proteins. In particular, S307 is a commonly targeted residue that correlates negatively with insulin sensitivity[8,9]. Phosphorylation of this residue disrupts the interaction of IRS1 with the IR. However, numerous other serine residues exist, including serine 302, 318, 612 and others, whose phosphorylation is caused by agents that regulate insulin sensitivity [82].

The Effects of FFAs on Insulin Resistance

Elevated serum FFAs, commonly observed in obese individuals[83,84], are linked to numerous disease including type 2 diabetes, hypertension, dyslipidaemia, hyperuricaemia and abnormal fibrinolysis[85-87]. In fact, elevated serum FFAs are often indicative of insulin sensitivity in diabetic individuals[88,89]. Further highlighting the link between obesity-elevated serum FFAs and insulin resistance is the fact that over 80% of type 2 diabetics are obese and have elevated serum FFAs[90].

Interestingly, lipid infusion into healthy humans for 3-4 hours causes whole body insulin resistance[91]. The effects of these lipids on whole body insulin sensitivity is mediated by slightly different mechanisms in the liver and muscle tissues[92]. FFA accumulation in muscle, by either increased FFA uptake and/or decreased mitochondrial FFA oxidation, decreases insulin stimulated tyrosine phosphorylation of

IRS1, PI3K activity and insulin stimulated GLUT4 translocation[64]. As mentioned earlier, insulin stimulated tyrosine phosphorylation of IRS1 by the IR is reduced by agents that cause insulin resistance. Effectively, the serine phosphorylation reduces insulin signaling by inhibiting the association of IRS1 with the IR[8,9]. In muscle, studies suggest that protein kinase theta (PCKθ) phosphorylates IRS1 in response to FFA accumulation[85]. In agreement, knockout of PKCθ prevented the FFA induced reductions of both IRS1 tyrosine phosphorylation and PI3K activity[80].

FFAs cause insulin resistance by a slightly different mechanism in hepatocytes. Interestingly, the infusion of FFAs into healthy humans increases liver glucose production during hyperglycemic, insulinopenic clamps[93]. However, this effect was lost in individuals fasted overnight[94,95]. Further work demonstrated that elevated FFAs induced insulin secretion in fasted individuals, thus countering the effects of FFAs on gluconeogenesis. Hence, FFAs were able to increase hepatic glucose production in fasted individuals by maintaining fasting insulin levels in humans with a somatostatin-insulin infusion[96].

Similar results were observed in rats fed a high fat diet for only 3 days in which hepatic lipid accumulation caused liver, but not muscle insulin resistance[97]. In these studies, PKCε negatively regulated the IR tyrosine kinase activity and thus the tyrosine phosphorylation of IRS2[97]. Although, the mechanistic data presented in these studies are correlative, the role of PKCε appears correct, as anti sense attenuation of PKCε expression blocked the ability of fatty acids to suppress insulin signaling despite 3 days

of high fat diet[98]. Thus, although FFAs cause insulin resistance in hepatocytes and muscle tissues, different PKC isoforms mediate the effect.

Overall, the data suggests that elevated serum FFAs contribute to the development of insulin resistance. However, data in humans suggests that FFAs only partially drive insulin resistance. In agreement, elevated serum FFAs fail to correlate with insulin resistance and enhanced hepatic glucose output in non-diabetic obese humans as they do in type 2 diabetics[99]. Additionally, studies in rats utilizing anti-lipolytic agents potently lowered serum FFA levels, blood glucose levels and improved insulin sensitivity[100]. However, in humans different results were obtained. Treatment with Acipomox, an anti-lipolytic agent, decreased serum FFA levels and improved insulin sensitivity in obese non-diabetic subjects. On the other hand, in obese diabetic individuals, Acipomox failed to substantially improve insulin sensitivity, despite reducing serum FFAs[101]. Thus, although the accumulation of FFA in peripheral tissues plays a significant role in insulin resistance, additional factors must also contribute to insulin resistance in humans.

Inflammation and Metabolic Disease

Over a century ago, high doses of anti-inflammatory sodium salicylate were demonstrated to reduce glucose secretion in the urine of patients who were likely type 2 diabetic and insulin resistant[74,102,103]. This effect of salicylates was rediscovered in 1957 when a high dose of aspirin was used to treat an insulin dependent diabetic for arthritis associated rheumatic fever. Amazingly, the patient became euglycaemic and no longer needed insulin. However, upon discontinuation of aspirin treatment the patient's ability to regulate blood glucose levels reversed to pretreatment conditions[104]. A mechanistic

understanding of how salicylates regulated glucose disposal was not available as these early studies focused on insulin secretion and not on insulin resistance[74,105]. Not until the start of the 21st century were salicylates shown to mediate their effects on insulin sensitivity by inhibiting the inhibitory κ B Kinase β (IKKβ)[106,107]. Since these studies, numerous groups have demonstrated the correlation between inflammatory mediators and insulin resistance in humans[108,109]. Furthermore, by utilizing rodent models, the role of inflammation in the development of insulin resistance is now beginning to be understood[74,77].

The Effects of Cytokines on Insulin Resistance

The discovery that tumor necrosis factor α (TNFα) was over expressed in rodent models of obesity and in humans provided the first mechanistic link between obesity and inflammation[110-112]. Later work confirmed that TNFα impaired insulin signaling in adipocytes by decreasing IR and IRS tyrosine phosphorylation[113,114]. Furthermore, neutralization of TNFα in obese rats improved insulin sensitivity and lowered plasma glucose and lipid levels[115], while deletion of TNFα[116], or the TNFα receptors[117] in mice protected against diet induced obesity and insulin resistance. Surprisingly, the same results were not observed in humans as early studies neutralizing TNFα failed to improve insulin sensitivity[118,119]. These studies were limited by small sample sizes, single injections and a failure to demonstrate that the antibodies properly attenuated TNFα action. Since these studies, patients receiving regular TNFα neutralization to combat rheumatoid arthritis demonstrated improved glycaemia which reverted upon the completion of treatment[120,121].

The regulation of insulin signaling is not the only mechanism by which TNFα drives insulin resistance. TNFα also functions to lower the transcriptional levels of mRNAs associated with improved insulin sensitivity and lipogenesis in adipocytes and hepatocytes[122,123]. These studies demonstrated that TNFα infusion favors the expression of lipolytic and cytokine gene expression in adipose tissue, while favoring genes involved in cholesterol and lipid synthesis in hepatocytes[123]. Furthermore, TNFα robustly stimulates lipolysis, causing an enhancement of serum FFAs[124,125]. Despite the potent effects of TNFα on muscle insulin sensitivity, few changes were observed in muscle gene expression[114,115,123].

Since the identification of TNFα as a mediator of insulin resistance, investigators have identified other cytokines that modulate insulin action. These include, interleukin 1 beta (IL-1β), interleukin 6 (IL-6), monocyte chemo-attractant protein 1 (MCP1), and others (for complete review see[126]). Through diverse mechanisms, these cytokines regulate insulin signaling and insulin sensitivity in rodent models.

IL-1β is one of the least studied cytokines in terms of its regulation of insulin signaling and cell metabolism. Similarly to TNFα, IL-1β is produced by monocytes and macrophages, and increases in expression during obesity[127]. The expression level of IL-1β also correlates well with insulin resistance[128]. More recently, investigators have examined the effects of IL-1β on insulin signaling and metabolism in cultured cell models. Interestingly, IL-1β also negatively regulates insulin stimulated glucose transport in cultured adipocytes. Unlike TNFα, IL-1β seems to affect both the tyrosine phosphorylation and the expression levels of IRS-1 but not IRS-2. IL-1β also does not

alter adipocyte gene expression significantly. The levels of the adipocyte transcription factors peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα) remain unchanged despite IL-1β treatment[129,130]. Thus, in adipocytes it appears that IL-1β utilizes slightly different mechanisms to impair insulin sensitivity than TNFα.

IL-6 is the most controversial cytokine in terms of its role in insulin resistance. Studies in hepatocytes demonstrated that IL-6 is capable of suppressing insulin signaling by inducing the suppressor of cytokine signaling 3 protein[131]. Interestingly, IL-6 functions as a potent lipolytic agent in adipose tissue, possibly to increase FFA availability for muscle oxidation during exercise[132,133]. In elderly human muscle, IL-6 administration actually increased glucose uptake[133]. However, acute administration of IL-6 to mice decreases insulin stimulated glucose uptake in skeletal muscle, and increases hepatic glucose output[134]. The opposite effect is observed in IL-6 deficient mice which become obese and insulin resistant, perhaps due to increased leptin and TNFα levels, and potential leptin resistance that is partially reversed through IL-6 administration[135]. Conversely, over expression of IL-6 in mice causes insulin resistance and obesity[136]. Thus more work is required to identify the exact role of IL-6 on insulin sensitivity.

Recent work has highlighted the importance of MCP-1 in obesity and insulin resistance. In adipose tissue, the production of MCP-1 correlates remarkably well with obesity, resulting in the recruitment of macrophages to adipose tissue in both humans and in mice[137-140]. In agreement, the number of macrophages in adipose tissue

increases from 5-10% in lean, to over 50% in obese individuals[141]. Interestingly, the macrophage content of adipose tissue is a dynamically regulated process as weight loss following bariatric surgery is followed by equivalent losses of adipose tissue macrophages[142]. In mice, the over expression of MCP-1, specifically in adipose tissue, increases adipose macrophage infiltration, serum FFA levels and decreases insulin sensitivity in liver and skeletal muscle[143,144]. Interestingly, the genetic deletion of MCP-1 fails to prevent diet induced obesity and insulin resistance[145] while the deletion of the MCP-1 receptor did prevent insulin resistance, macrophage accumulation in adipose tissue and preserves insulin sensitivity[146]. These discrepancy may be due to other MCP family ligands (MCP-2, 3, 4 or 5) which may bind the MCP-1 receptor and may stimulate the macrophage recruitment to adipose tissue[147-150].

Other cytokines and inflammatory mediators undoubtedly influence insulin signaling and metabolism in the peripheral tissues and cells. These include IL-18, monocyte colony stimulating factor, plasminogen activator inhibitor 1, C-reactive protein and others. However, their roles remain less well defined. Without question, more cytokines remain to be identified that will play a role in insulin signaling and peripheral tissue glucose metabolism.

Promotion of Insulin Sensitivity and Resistance by Adipokines

It is commonly recognized now that adipose tissue functions as a secretory organ that produces both cytokines, such as TNFα, that cause insulin resistance, and beneficial agents that improve insulin sensitivity. Leptin was the first adipose specific secretory protein discovered to have an effect on whole body metabolism[151]. Since then numerous adipokines (adipocytes derived cytokines) have been identified[152,153].

Remarkably, leptin regulates whole body metabolism by reducing food intake while simultaneously increasing energy expenditure through hypothalamic sympathetic nervous signaling[154,155]. Furthermore, leptin expression increases dramatically in obesity suggesting that increased leptin secretion during obesity may function as a negative feedback mechanism to reduce adiposity[156,157]. Despite this elegant mechanism to control adiposity, resistance to the actions of leptin develops during obesity.

Unlike leptin expression, the expression of adiponectin decreases during obesity[158,159]. Interestingly, adiponectin's effects are pleiotropic as exogenous adiponectin lowers hepatic glucose production[160,161], increases muscle glucose uptake and fatty oxidation[162], decreases body weight and adipose mass through neuronal regulation[163], decreases serum FFA levels[164] lowers blood pressure[165] and protects against myocardial infarction[166,167]. To mediate these functions, adiponectin activates the 5'amp activated protien kinase (AMPK)[167-169]. AMPK regulates numerous intracellular substrates to produce a net effect which includes increased muscle FFA oxidation and glucose uptake, increased liver FFA oxidation, decreased gluconeogenesis, decreased lipogenesis and cholesterol synthesis and FFA release from adipose tissue.[170]

Other adipokines exist that effect insulin resistance and glucose metabolism. One example is resistin which results in acute hepatic, but not peripheral insulin resistance[171]. Gene deletion of resistin lowers hepatic glucose production and fasting blood glucose levels[172] while overexpression of resistin increases fasting blood glucose levels and decreases glucose tolerance[173]. However, resistin may not be a true

adipokine as in humans, resistin is produced primarily by the stromal vascular cells of adipose tissue[174], raising questions as to whether resistin is a true adipokine or actually a cytokine. A more recently described adipokine is retinol binding protein 4 (RBP4). Interestingly, the elevation of serum RBP4 levels in mice causes insulin resistance while decreasing RBP4 function enhances insulin sensitivity[175]. Because of the effects on whole body insulin sensitivity, the cellular functions of RBP4 remain an area of active research.

Overall, the adipokines and cytokines described above greatly influence insulin sensitivity and cellular metabolism. Interestingly, both macrophages and adipocytes produce many of these molecules to influence whole body glucose homeostasis. Therefore, studies of adipocyte and macrophage function can offer insights into improving metabolic disease.

The Mediation of Insulin Resistance by Inflammatory Signaling

Inflammation is mediated primarily by two major signaling conduits, the JNK1/2 and nuclear factor κ B (NFκB) pathways. Other pathways are also activated by inflammatory stimuli, such as the p38 stress activated protein kinases (p38 SAPK), ERK1/2, aPKC and others. However, JNK and NFκB represent the two most studied, and perhaps potent, inflammatory signaling cascades in terms of insulin signaling regulation.

JNK and Insulin Resistance

The JNK family of protein kinases, phosphorylate the activator protein 1 (AP-1) transcription factor family in response to intracellular stresses[176]. These stresses, including ultra-violet light, reactive oxygen species, certain cytokines and osmotic stress activate JNK through a complex series of cell and stimulus specific events. Typically, the action of JNK is similar to ERK activation as a number of upstream kinases initiate a phosphorylation cascade that ultimately leads to JNK phosphorylation and activation. The kinases, MAPK Kinases 4 and 7(MKK4 and MKK7), phosphorylate the activation loop of JNK, preferentially on a tyrosine and threonine residue respectively[177,178]. However, despite this preference, both kinases are capable of phosphorylating the tyrosine and threonine residue. This phosphorylation event culminates with JNK activation and phosphorylation of targets such as cJUN, ATF-2, BCL-2 and other transcription factors and cytosolic protein targets[176]. Inactivation of JNK is achieved through dephosphorylation mediated by serine, threonine and dual specificity phosphatases that dephosphorylate both the threonine and tyrosine residues[179].

Upstream of MKK4 and MKK7 a more complex picture emerges as numerous upstream kinases including transforming growth factor-β activated kinase, the mixed lineage kinases, the activator of S-phase kinases and the MAPK/extracellular regulated kinases can activate MKK4 and MKK7[180]. These upstream MAPKKK are capable of being activated by tyrosine kinase receptors through RHO GTPases[181,182], and through interactions with receptor adaptor proteins such as TNFα receptor associated factor 2[180]. Interestingly, the sterile 20 protein kinase family, and in particular MAP4K4/NIK

has been implicated in JNK activation and will be discussed in more detail later[183,184]. Further complicating the issue, JNK activating signaling cascades assemble on numerous scaffolding proteins, depending on the stimulus, to create local JNK signaling environments with in the cell[185]. Thus, considering the vast number of potential upstream kinases and scaffolding proteins that potentially regulate JNK, understanding the regulation JNK in response to various stimuli can be complex.

As mentioned earlier, in obesity the expression of inflammatory cytokines like TNFα increases in adipose tissue[110,111] which are capable of increasing JNK activity. In diet induced and genetic leptin deficient (*ob*/ob) obese mice, JNK activity is increased[186]. Interestingly, both IRS1 and IRS2 possess JNK binding domains, and are capable of being phosphorylated by JNK. Similar to PKCθ, JNK readily phosphorylates IRS1 at S307, resulting in the disruption of the IR and IRS interaction and preventing the tyrosine phosphorylation of IRS1[78]. This phosphorylation event is mediated by JNK1, as JNK1 deficient rodents, but not JNK2 null mice were protected from high fat diet induce insulin resistance and IRS1 S307 phosphorylaiton[186]. Furthermore, these JNK1 deficient mice were protected from high fat diet induced obesity and adipose tissue expansion. Interestingly, JNK1 deficiency, in hematopoietic cells, protects mice from the induction of inflammatory cytokines (TNFα, IL-6 etc.) commonly observed from high fat diet treatment, without effecting weight gain or adiposity. Conversely, in these same studies the authors show that JNK1 deficiency in non-hematopoietic cells protects mice from the adipose tissue expansion in response to a high fat diet, thus keeping the mice leaner[187]. Hence, JNK signaling plays a significant role mediating the development of obesity and obesity derived inflammation and insulin resistance.

Furthermore, enhanced JNK signaling promotes adipose dysfunction and macrophage inflammation in obesity.

IKKβ and NFκB Induced Insulin Resistance

The NFκB family of transcription factors consists of 5 members noted, p65(RelA), RelB, c-Rel, p50 and p52. In the canonical NFκB signaling pathway, inflammatory stimuli, such as tumor necrosis factor α (TNFα), activate IKKβ which subsequently phosphorylates the inhibitor-κ B protein (IκB). Following, IKKβ phosphorylation, IκB undergoes proteasomal degradation, freeing the NFκB transcription factors p65/p50 for nuclear localization and the transcription of cytokines, pro and anti-apoptotic factors, growth factors and cellular adhesion molecules[188]. IKKβ exists in a complex with the highly homologous IKKα and the non-catalytic IKKγ subunits[189]. IKKα activates a second NFκB pathway dubbed the alternative pathway by phosphorylating p100. Following phosphorylation, p100 is ubiquitinated and partially degraded by the proteasome, producing p52[190]. The active RelB/p52 heterodimer translocates to the nucleus inducing transcription of NFκB target genes that are important in B cell and secondary lymphoid organ development.

Unlike IKKα, IKKβ plays a central role in the development of insulin resistance. Interestingly, salicylate treatment improves insulin sensitivity in rodent models of obesity and insulin resistance through IKKβ inhibition[106]. In these same studies, the authors showed that the heterozygous deletion of IKKβ protects against the development of insulin resistance in high fat diet and leptin deficient mice. Furthermore, heterozygous deletion of IKKβ also protects from the negative effects of acute FFA on insulin

sensitivity[191]. One postulated mechanism is the phosphorylation of IRS-1 on S307[79,192,193]. In IKKα/β null embryonic fibroblasts the phosphorylation of IRS-1 S307 is reduced[193]. However, the *in vivo* relevance of this interaction is questionable, as expression of a dominant inhibitory IκBα mutant in hepatocytes completely prevented the insulin resistance caused by liver specific constitutive IKKβ activation[194]. In these same double transgenic mice, the authors also show that inflammatory cytokine expression, such as IL-6, is reduced suggesting that the primary effect of IKKβ inhibition in the liver is the prevention of inflammatory gene expression[74]. NFκB also mediates TNFα repression of lipogenic gene expression and the enhancement of cytokine expression in adipocytes[122]. Additionally, deletion of IKKβ in myeloid cells prevents systemic insulin resistance and maintains glucose sensitivity in rodents on a high fat diet[195]. In these same studies, knockout of IKKβ, specifically in hepatocytes maintains liver insulin sensitivity despite the development of insulin resistance in the muscle of diet induced obese mice. This suggests that inactivation of IKKβ in the liver affects only local insulin sensitivity. Thus, IKKβ represents a crucial kinase in the regulation of systemic and local tissue insulin resistance.

Toll Like Receptor Mediated Inflammation

The Toll-like receptor (TLR) family represents a basic defense system of the innate immune system against infection. TLRs belong to a class of receptors that are stimulated by pathogen-associated molecular patterns [196]. The first TLR discovered was shown to be essential for embryonic dorsal-ventral patterning in *Drosophila*[197]. Years later, the receptor was revealed to have an essential role in the innate immune

response of insects against fungal infections[198]. Since then 10 mammalian homologues, denoted TLR1-10, have been discovered that mediate key aspects of the mammalian innate immune response. These include the recognition of double stranded viral RNA by TLR3 and gaunosine/uridine rich viral single stranded RNA by TLR7/8. Additionally, TLR9 recognizes unmethylated cytosine and guanosine motifs in viral DNA, while bacterial peptidoglycan and lipoteichoic acid are recognized by TLR2/6 and bacterial flagellin by TLR5[199,200]. Although all of the TLRs activate a similar intracellular signaling cascade to stimulate cytokine and interferon production, the best characterization of these signaling events has been done in the lipopolysaccharide (LPS) sensing TLR4.

TLR4 Signaling

LPS consists of three components, lipid A, a core oligosaccharide and an O side chain of which the lipid A portion is the most potent stimulator of TLR4. Gram negative bacteria utilize LPS as an essential component of their cell wall, making the molecule a general marker of bacterial infection for the immune system[201]. LPS is unable to bind TLR4 alone and requires several interacting molecules including LPS binding protein (LBP), CD14 and lymphocyte antigen 96 (MD-2)[199]. Soluble LBP binds LPS and facilitates the association of LPS with CD14[202]. In turn, CD14 transfers LPS to TLR4/MD-2 to modulate LPS recognition[203]. Current data suggests that LPS cannot bind TLR4 directly and that the association of these intermediates are necessary for TLR4 activation by LPS[199].

Upon LPS binding, TLR4 oligomerizes and recruits adaptor proteins through interactions with its Toll-interleukin-1 receptor (TIR) domain[200]. The TIR domain of

TLR4 is essential for an LPS response as single missense mutation in the TIR domain of TLR4 renders C3H/HeJ mice insensitive to LPS[204]. Five TIR domain-containing adaptor proteins interact with TLRs. These include myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing interferon β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α and HEAT-Armadillo motifs-containing protein (SARM)[205]. The combination of these adaptor proteins determines the downstream signaling cascades activated. Interestingly, the only TLR to utilize all of these adaptors is TLR4[199].

The association of TIRAP and MyD88 with TLR4 is critical for LPS induced septic shock. TIRAP contains a PIP2 binding domain that facilitates recruitment to the plasma membrane. TIRAP then aids in the association of TLR4 with MyD88[206]. Downstream of MyD88, the adaptor protein TNF receptor-associated factor 6 (TRAF6) is recruited through interactions with the IL-1 receptor associated kinase 1 (IRAK-1). IRAK-1 is recruited by MyD88 and phosphorylated by IRAK-4 resulting in TRAF6 recruitment[200]. Interestingly, IRAK-4 knockout mice show LPS resistance and a substantial reduction in inflammatory cytokine expression[207]. TRAF6 complexes with ubiquitin-conjugating enzyme 13 (UBC13) and the ubiquitin-conjugating enzyme E2 variant 1, isoform A (UBEV1A). Together these enzymes activate the transforming growth factor-β kinase (TAK1). TAK1 is capable of activating IKKβ and NFκB along with MAP kinase signaling cascades that activate AP-1 transcription[199]. Additionally, the interferon regulatory factor 5 (IRF5) is activated by MyD88 and appears important for the induction of a subset of cytokines including TNFα and IL-6. Furthermore, the deletion of MyD88 in mice also reduces LPS induced inflammatory cytokine production[208]. Macrophages

from these mice have intact, but slightly delayed MAPK and NFκB activation, suggesting that other MyD88 independent pathways must be activated by TLR4.

Interestingly, defects in the late phase activation of NFκB and MAPKs were observed in TRIF deficient macrophages, suggesting that TRIF activated the delayed MAPK and NFκB responses observed in the absence of MyD88. The combined deletion of TRIF and MyD88 severely blunted NFκB and MAPK activation[209]. Thus, it is the combined signaling of MyD88 and TRIF that controls the TLR4 response to LPS. TRIF mediates signaling activation by associating with the serine/threonine kinase receptor interacting protein 1(RIP1) which activates NFκB and MAPK signaling. Additionally, TRIF appears important for the activation of the Type 1 interferon response by interferon regulatory factor 3 (IRF3). IRF3 is activated by the recruitment of TRAF3. TRAF3 associates with the TRAF family member associated NFκB activator (TANK), TANK binding kinase 1 (TBK1) and IKKε to activate downstream signaling. Interestingly, TBK1 and IKKε are critical for the activation of IRF3 and the induction of the Type 1 interferon response.

TLR 4 Stimulation of TNFα Transcription

Although numerous cytokines are produced by TLR4 activation, much focus has concentrated on the induction of TNFα by LPS. Activation of TNFα gene expression by LPS appears mediated by a transcription factor complex which includes Egr-1, Sp1, ATF2/cJUN, ETS, and ELK in conjunction with the CBP/p300 coactivator proteins [210,211]. The role of NFκB appears more controversial as some data support a role for NFκB in driving partial TNFα expression[212,213]. However, recent studies demonstrate that role of

NFκB may be a secondary maintenance event for TNFα mRNA levels as the initial induction of the TNFα promoter does not change in response to chemical and genetic inhibitors of NFκB[214].

TLR 4 in Metabolic Disease

Interestingly, LPS is not the only ligand that induces TLR4 mediated TNFα transcription, as endogenous molecules, such as saturated fatty acids and heat shock protein 60, along with drugs such as Taxol are also capable [215-218]. Circulating FFA, whose levels are increased during obesity, due to enhanced release from the enlarged mass of adipose tissue, are proposed to suppress insulin action as discussed previously. Reports indicate that FFA released from adipocytes activate inflammatory cytokine production in macrophages. Supporting this hypothesis, co-culture models of adipocytes and macrophages enhance macrophage inflammatory cytokine expression[219,220]. However, it is unclear if the macrophages were activated by FFA or adipose adipokine/cytokine secretion in these studies. In various cell culture models, palmitate and laurate induce TNFα transcription in macrophage cell lines[218,220]. Furthermore, TLR4 null mice are protected from high fat diet induced insulin resistance because of decreases in inflammatory gene expression in macrophages [221]. However, these studies with TLR4 null mice failed to adequately consider the effects of potential contaminating LPS. Emerging data suggests that mice with compromised TLR4 and deletion of CD14 may actually be more sensitive to the effects of high fat diet (personal communication from Dr. Silvia Corvera). Thus the exact role of TLR4 in metabolic disease remains to be fully defined.

Model Systems for Studying Inflammation and Insulin Resistance

Insulin alters the metabolic activities of liver, muscle and adipose tissues. Therefore, cell lines derived from all of these tissues are potentially interesting model systems to study insulin signaling and insulin resistance. However, the focus of these studies is the adipocyte and macrophage, as both cell types have great influence on the development of insulin resistance and inflammation as will be discussed below. A greater understanding of the physiological processes in these cell types will enhance our knowledge of inflammation and insulin resistance.

The Adipocyte as a Model System

The importance of adipocytes in driving systemic glucose homeostasis is highlighted by the fact lipodystrophy, or a complete absence of adipose tissue, causes insulin resistance in both humans and mice[222-227]. To maintain glucose homeostasis, adipose tissue sequesters FFAs in the circulation and produces insulin sensitizing adipokines. Dysfunction of these adipose processes results in whole body glucose intolerance and insulin resistance[63]. Highlighting the importance of healthy functioning adipocytes, selective loss of GLUT4 in adipocytes causes systemic insulin resistance while enhanced GLUT4 expression in adipocytes causes enhanced insulin sensitivity and protects against high fat diet induced insulin resistance[26,29]. Furthermore, drugs which enhance adipose lipid storage capacity, such as thiazolidinediones, improve insulin sensitivity in diabetic patients[228].

Normally during fed periods, adipocytes take up glucose and lipids and synthesize triglyceride for future energy needs. Upon fasting, adipocytes readily hydrolyze the triglycerides to produce FFA and glycerol to be utilized as an energy

source by the peripheral tissues[63,229]. Upon the development of obesity, adipose tissue release excess lipid in the form of FFA due to increased lipolysis. The increase in FFA release is caused by a chronic state of inflammation that develop as adipocytes in obese individuals secrete cytokines like MCP-1, resulting in macrophage recruitment to adipose tissue[141]. As macrophages accumulate in the adipose tissue, the local concentrations of cytokines, such as TNFα and IL-1β increase, enhancing lipid efflux from the adipocytes[114,129,130,230]. Additionally, adipocytes also contribute to the chronic inflammatory state by also secreting cytokines and thus further activating lipolysis in a paracrine manner. The result of adipose dysfunction is a positive regulatory loop with macrophages that enhances inflammation and exacerbates serum FFA concentrations[63]. As mentioned earlier, the increase in serum FFA levels causes skeletal muscle and liver insulin resistance. Thus, enhancing adipose tissue lipid storage capacity is a logical strategy for curbing insulin resistance.

One logical target for curbing adipose tissue inflammation is PPARγ. PPARγ is considered the master regulator of adipogenesis and lipogenic enzyme expression[231]. As mentioned earlier, TNFα and other cytokine's suppress PPARγ transcription and thus the expression of lipogenic enzymes. Treatment of insulin resistant individuals with drugs that activate PPARγ, such as thiazolidinediones enhance insulin sensitivity by increasing adipose FFA storage capacity and decreasing inflammation[232]. Hence, mechanisms that enhance PPARγ activity and adipose function are likely to promote whole body insulin sensitivity.

The Role of Macrophages in General and Obesity Derived Inflammation

Macrophages are important immune cells involved in processes such as wound healing and protection from bacterial infections. Macrophage populations are distributed throughout the body and help maintain healthy tissue function[233]. The primary role of macrophages is to phagocytose pathogens or cellular debris and activate other immune cells in response to infection[234]. Because of their broad tissue distribution, macrophages are capable of acting as a sensor of infection and mounting a potent inflammatory response. An excellent example of this is the activation of macrophage TLR4 by LPS which causes a robust increase in TNFα secretion.

Macrophages also regulate less intense and milder forms of inflammation. As discussed earlier, hints regarding the role of inflammation in insulin resistance had been known for over a century[102]. The concept of obesity induced inflammation was first demonstrated fifteen years ago, when increased production of TNFα from adipose tissue was observed in obese rodents[111], and later humans[110]. Initially, the adipocyte was thought to be the major source of the obesity induced inflammation. Subsequent studies clearly demonstrated that the inflammatory cytokines are also being produced by non-adipocytes in the stromal vascular cell fractions[235] . Further studies revealed that macrophage infiltration into adipose tissue is a major mechanism driving inflammation upon the development of obesity[138,141]. Since these studies, macrophage mediated inflammation in obesity has been shown to play a role in numerous diverse diseases, including Alzheimers[236], psorisis[237], certain cancers[238,239] and atherosclerosis[240].

The infiltration of adipose tissue by macrophages is an important developmental stage in insulin resistance. Treatments, such as thiazolidinediones, promote insulin sensitivity by increasing adipocyte lipid storage capacity and suppressing adipocyte inflammatory gene expression. However, these treatments also reduce adipose tissue macrophage content in humans and rodents[138,241]. Interestingly, the decrease in adipose macrophage content is accompanied by a corresponding decrease in inflammatory gene expression. Also, as discussed earlier, it is the production of these cytokines that drives insulin resistance by two mechanisms, the stimulation of adipose FFA release, and direct negative effects on insulin signaling networks[63]. Thus, the macrophage represents an important target for attenuating adipose inflammation and consequently, whole body insulin resistance. Therefore, a better understanding of the cellular mechanisms that regulate macrophage inflammation is necessary.

RNAi Therapeutics for Inflammatory Disease

RNA interference (RNAi) is an evolutionary conserved cellular mechanism for quelling gene expression that is broadly conserved from plants, fungi, insects to mammals[242-246]. Following the discovery of RNAi in 1998[242], the dream of a potential therapeutic application of RNAi became real upon the discovery that RNAi was functional in mammals[245,246]. Since then, numerous studies have attempted to harness the power of RNAi to treat numerous diseases, including cancer, viral hepatitis, hypercholesterolemia, HIV, sepsis and inflammation in mice[246-251] and nonhuman primates[252]. The reason for the excitement regarding the therapeutic potential of RNAi

is that for the first time every gene expressed in mammalian cells can be targeted for suppression.

The Biology of RNAi

RNAi is mediated by small (21-25 nucleotide) double stranded RNA molecules, termed small interfering RNAs (siRNAs)[253-256]. siRNAs, either endogenously derived from longer double stranded RNAs (dsRNAs) or from exogenously delivery, contain 2-3 nucleotide overhangs on the 3' strand. Endogenously derived siRNA are produced by the RNAse III-like enzyme, Dicer[257,258], and loaded into an RNAi induced silencing complex (RISC) containing the dsRNA binding protein R2D2 and Dicer-2 enzyme. The R2D2 protein binds to the more thermodynamically stable end to ensure loading of the correct antisense sequence into the RISC complex[259]. Interestingly, the strand with a lower 5' thermodynamic stability is preferentially loaded into the RISC[260,261]. Upon loading, R2D2 is substituted for the RNAi endonuclease Argonaute-2 (AGO-2), which appears to be the functional RNAi endonuclease in the RISC[259,262,263]. It is worth mentioning here that this same complex appears to regulate a similar mechanism of silencing with incomplete matches from endogenously derived microRNAs (miRNAs). Despite being produced from different precursors, miRNAs and siRNAs appear to be interchangeable as they both can suppress gene expression in cells. However, whereas siRNAs generate cleavage of the mRNA target, miRNAs suppress translation by an unknown mechanism[264,265]. Furthermore, in unique circumstances, miRNAs can also augment translation.

Although, RNAi offers great therapeutic potential, numerous obstacles exist in the application of RNAi as a disease intervention. These obstacles include rapid

degradation of siRNA oligonucleotides in the serum and the extracellular environments, rapid excretion through the kidney, and low permeability through tight junctions and across cell surface membranes[266,267]. Although, some success has allowed for a few clinical trials[268], new techniques are needed for the delivery of RNAi to specific target tissues and cell types. Furthermore, the development of an orally deliverable RNAi therapeutic would be the "holy grail" of the blooming field of RNAi therapeutics.

MAP4K4 as a Therapeutic Target

MAP4K4 is a member of the germinal center kinase (GCK) family of the sterile 20 protein (STE20p) kinases. Originally, MAP4K4 was identified as an interacting molecule with the receptor tyrosine kinase adaptor protein, NCK adaptor protein 1(NCK)[269]. Through *in vitro* overexpression studies, the authors demonstrated that MAP4K4 may activate an MEKK1, MKK4 and JNK cellular signaling cascade. The activation of JNK by MAP4K4 in TNFα signaling was later confirmed in human cell lines. However, in these studies the authors did not see a requirement of MEKK1 for JNK activation, perhaps because the human MAP4K4 isoform used in these studies did not contain the proline rich regions thought to interact with the SH3 domains of NCK[183].

Later studies suggested a prominent role for MAP4K4 in development, as *Drosophila* expressing mutant MAP4K4 homologues failed to undergo dorsal closure[270], and because deletion of MAP4K4 in mice prevented the migration of the mesoderm during gastrulation[271]. Interestingly, the role for MAP4K4 in development appears to be mediated by ephrin receptor signaling[184]. Further studies indicated that MAP4K4 was mediating cell migration, as attenuation of MAP4K4 by siRNA greatly reduced the ability of multiple cancer cell lines to migrate[272], while other studies demonstrated a role for

MAP4K4 in lamellipodia formation and actin rearrangement[273]. Despite all of the earlier studies indicating that MAP4K4 functioned upstream of JNK, later studies showed that the defect observed in the epithelial to mesodermal transition during embryogenesis was due to activation of p38 by MAP4K4 and not JNK[271,274]. However, because of the lethality of the MAP4K4 null mouse, no insight into the role of MAP4K4 beyond embryogenesis was obtained.

A role for MAP4K4 in the metabolic regulation of adipocytes was recently discovered. Through an siRNA screen for kinases that potentially regulate glucose uptake and adipocyte function, MAP4K4 was discovered to be a negative regulator of adipogenesis[275]. In these studies, attenuation of MAP4K4 by siRNA was shown to increase PPARγ expression, triglyceride storage and increase insulin stimulated glucose transport in adipocytes (see Figure 1.3 for model). Because earlier studies linked MAP4K4 to TNFα activation of JNK, the effects of TNFα were tested in adipocytes where MAP4K4 expression was silenced. Silencing of MAP4K4 partially protected from the negative effects of TNFα on PPARγ and GLUT4 gene expression[275], suggesting that MAP4K4 mediates some of the TNFα effects on adipocytes. Further studies, in muscle, showed that MAP4K4 could activate ERK1/2 in human muscle explants and that silencing MAP4K4 in these muscle explants protects from TNFα induced insulin resistance[276].

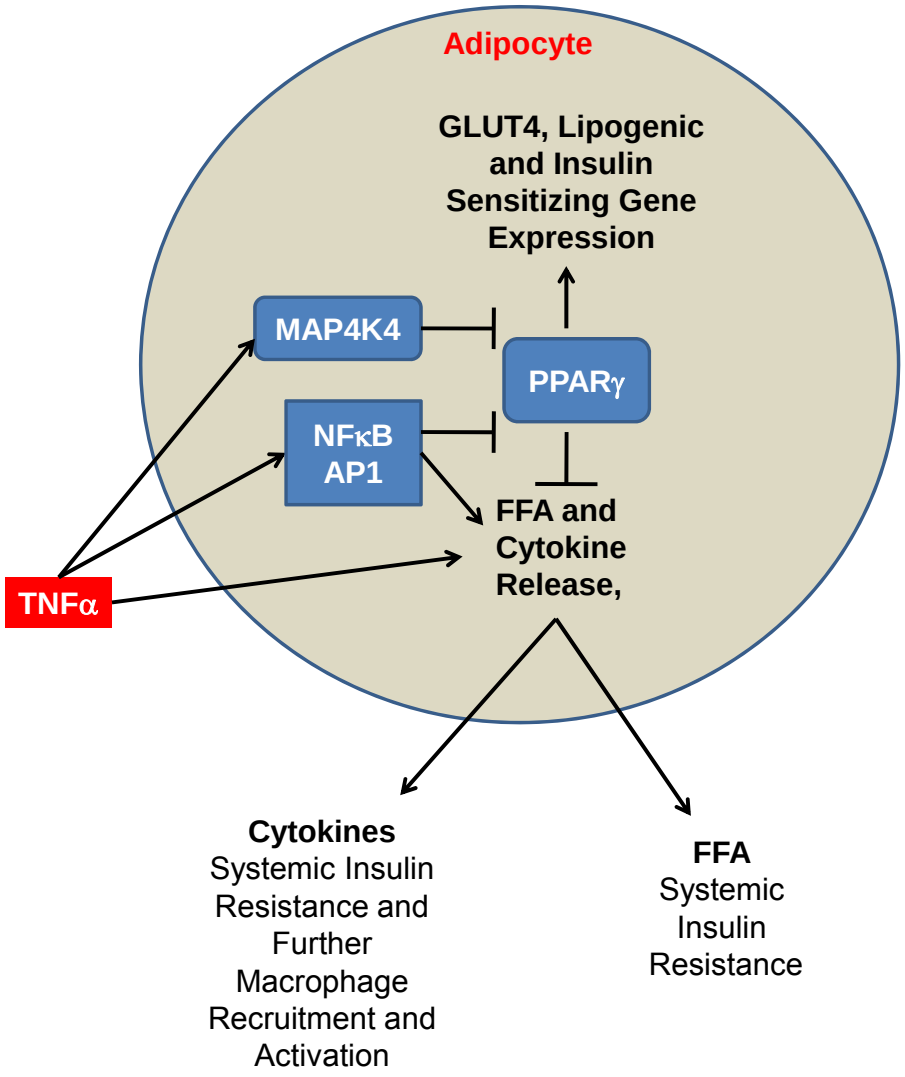

Figure 1.3 Model for MAP4K4 function in adipocytes. TNFα activates numerous signaling pathways including NFκB and AP-1 gene transcription. Additionally, MAP4K4 functions in a separate pathway to suppress PPARγ expression. The net result of these pathways is a decrease in the expression of genes such as GLUT4 and lipogenic genes which enhance adipose tissue function and triglyceride storage capacity. Additionally, AP-1 and NFκB promote inflammatory gene expression along with NFκB inhibition of PPARγ function which further suppresses lipogenic genes and results in FFA release. The decreased adipose tissue function and increased cytokine and FFA release help drive systemic insulin resistance.

Additionally, a role for MAP4K4 in mediating inflammatory effects also exists as silencing of MAP4K4 in primary mouse T-cells prevents their activation[277]. In these studies, the authors demonstrated that in co-culture models of activation, MAP4K4 was necessary for full activation of the TNFα promoter in T-cells. However, the mechanism by which MAP4K4 controlled TNFα promoter activation was not fully characterized. Never the less, this data does suggest a role for the MAP4K4 regulation of TNFα production in immunological cells. Furthermore, considering that TNFα is a major component of insulin resistance, MAP4K4 presents a potentially interesting target for attenuating metabolic disease.

Specific Aims

Initial studies from our laboratory demonstrated a role for MAP4K4 as a negative regulator of adipocyte function[275]. In these studies, MAP4K4 mediated part of the effects of TNFα on adipocytes. Additionally, the authors also observed that MAP4K4 expression was increased significantly following TNFα treatment in adipocytes[275]. Because little knowledge regarding the mechanisms by which TNFα controlled MAP4K4 expression existed, understanding the underlying signaling mechanisms regulating this process could potentially provide new therapeutic opportunities.

Earlier studies had also implicated MAP4K4 as a potential mediator of inflammatory signaling[183] and TNFα production[277]. Because of the importance of macrophages in driving the inflammatory response in multiple diseases, I wanted to determine in MAP4K4 functioned in a similar manner in macrophages *in vivo*. In order

to test this, we employed new strategies for attenuating MAP4K4 expression *in vivo* with RNAi. Thus, the specific aims of this study were;

1) To identify the signaling pathways by which TNFα regulates MAP4K4 expression.
2) To determine the role of MAP4K4 in LPS mediated macrophage inflammation *in vivo* and *in vitro.*

CHAPTER II: TNFα Stimulates Map4k4 Expression through TNFα Receptor 1 Signaling to cJUN and ATF2

Disclaimer: All experiments were performed by the author except for Figure 1A which was done by Xiaoqing Tang and Figure 2 which was done in collaboration with Adilson Guilherme and me. Anil Chawla prepared the GST fusion construct for antibody generation, while Andrea Hubbard, Kalyani Guntur and I all assisted in the testing and purification of the antibody from the serum. Xiaoqing Tang made the original observation that TNFα treatment increases MAP4K4 expression. I did all statistical analysis in this chapter.

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Abstract

TNFα is a cytokine secreted by macrophages and adipocytes that contributes to the low grade inflammation and insulin resistance observed in obesity. TNFα signaling decreases PPARγ and GLUT4 glucose transporter expression in adipocytes, impairing insulin action, and this is mediated in part by the yeast Ste20 protein kinase ortholog Map4k4. Here we show that Map4k4 expression is selectively upregulated by TNFα, while the expression of the protein kinases JNK1/2, ERK1/2, p38 SAP kinase, MKK4 and MKK7 shows little or no response. Furthermore, the cytokines IL-1β and IL-6 as

well as LPS fail to increase Map4k4 mRNA levels in cultured adipocytes under conditions where TNFα elicits a 3 fold effect. Using agonistic and antagonistic antibodies and siRNA against TNFα receptor 1 (TNFR1) and TNFα receptor 2 (TNFR2), we show that TNFR1, but not TNFR2 mediates the increase in Map4k4 expression. TNFR1, but not TNFR2, also mediates a potent effect of TNFα on the phosphorylation of JNK1/2 and p38 SAP kinase and their downstream transcription factor substrates cJUN and ATF2. RNAi-based depletion of cJUN and ATF2 attenuated TNFα action on Map4k4 mRNA expression. Consistent with this concept, the phosphorylation of ATF2, along with the expression and phosphorylation of cJUN by TNFα signaling, was more robust and prolonged compared to that of IL-1β, which failed to modulate Map4k4. These data reveal that TNFα selectively stimulates the expression of a key component of its own signaling pathway, Map4k4, through a TNFR1-dependent mechanism that targets the transcription factors cJUN and ATF2.

Introduction

Mitogen-activated protein (Map) kinases are cellular regulators of such diverse processes as apoptosis [278,279], differentiation [280-282] and proliferation [283]. Map kinase activation by a variety of extra-cellular stimuli can be mediated by many types of cell surface receptors and occurs through protein kinase phosphorylation cascades (for reviews see [278,283]). Such cascades involve the activation of a Map kinase kinase kinase (Map3k), which phosphorylates and activates a Map kinase kinase (Map2k)

which in turn phosphorylates and activates an effector Map kinase. This activation pattern is conserved from yeasts to mammals [284]. In the past few years, much effort has concentrated on identifying additional upstream kinases that regulate the downstream effector Map kinases. This effort has lead to the identification and characterization of the sterile 20 (Ste20p) family of protein kinases, which can act upstream of Map3ks. Ste20p kinases can be divided into two groups, the germinal center protein kinases (GCK) and the p21 activated protein kinases. One GCK member, which we recently identified as a negative regulator of adipogenesis, is Map4k4[275]. Map4k4 is a member of the GCK-IV group which appears to control cellular events ranging from cell motility, rearrangement of the cytoskeleton and cell proliferation [272,274,285-288]. The majority of studies focusing on Map4k4 propose that Map4k4 acts as an upstream activator of the cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), and p38 SAP kinase [272,274,288,289].

We initially identified Map4k4 in an RNAi screen for regulators of insulin-sensitive deoxyglucose uptake in 3T3-L1 adipocytes [275]. Remarkably, silencing of Map4k4 with siRNA caused an increase in the expression of peroxisome proliferator-activated receptor gamma (PPARγ), along with a corresponding increase in the expression of the insulin-responsive facilitative glucose transporter isoform 4 (GLUT4) [275]. PPARγ is nuclear hormone receptor that regulates the expression of numerous genes specific to the adipocyte differentiation program, including GLUT4 [290], which mediates insulin dependent glucose transport into both muscle and adipose tissue. Selective loss of GLUT4 in muscle or adipose tissue of mice leads to insulin resistance, glucose intolerance and diabetes [28,29]. Conversely, increased GLUT4 levels in the adipose

tissue of transgenic mice enhances whole body glucose homeostasis, suggesting that improved glucose disposal in adipose tissue may alleviate metabolic disease [26]. Thus, understanding the regulation of molecules such as Map4k4 that govern the expression and activity of PPARγ and GLUT4 in adipocytes may provide important insights into potential mechanisms that both promote and alleviate metabolic disease.

Our finding that Map4k4 acts as a negative regulator of adipogenesis and insulin stimulated glucose transport [275] suggested that it may play a role in signaling by tumor necrosis factor alpha (TNFα), a cytokine that is also a known negative regulator of adipogenesis and GLUT4 expression [122,123]. In agreement with this idea, Map4k4 has been suggested to be an upstream element in the TNFα signaling cascade [183]. Many studies have recently brought attention to the importance of cytokines in inducing the low grade inflammation observed in obesity [112,291], and in mediating biological effects antagonistic to insulin action in adipose tissue [113-115,292]. In agreement with this concept, TNFα levels are elevated in obesity and in diabetic mice [111,293] and humans [110,294,295]. Furthermore, TNFα attenuates metabolic signaling pathways initiated by the insulin receptor that lead to stimulation of glucose uptake [113,296] and inhibition of lipolysis [125]. RNAi-based silencing of Map4k4 partially protected against the TNFα induced depletion of both PPARγ and GLUT4 [275]. Additionally, siRNA silencing of Map4k4 has recently been shown to completely restore insulin sensitivity in muscle tissue from diabetic humans, in part by downregulating TNFα activation of JNK1/2 and ERK1/2 [289]. Hence, it appears that Map4k4 functions in the signaling pathways that mediate at least some of the inhibitory effects of TNFα on adipose and muscle tissue processes.

We previously noted that Map4k4 mRNA expression may be enhanced following TNFα treatment of 3T3-L1 adipocytes [275], a potentially significant modality through which TNFα may amplify it own acute signaling intensity. Based on this preliminary observation, the aim of the present studies was to further investigate whether Map4k4 protein kinase is indeed increased in adipocytes treated with TNFα and to identify the signaling pathways downstream of the TNFα receptors that control Map4k4 expression. We report here a remarkable specificity of TNFα action on Map4k4 protein kinase expression such that expression of other stress protein kinases such as JNK1/2 and p38 SAP kinase are unaffected by TNFα. Strikingly, TNFα action on Map4k4 is not mimicked by other cytokines such as IL-1β and IL-6. It is demonstrated here that this specificity of TNFα responsiveness appears due to a unique robust and prolonged phosphorylation of JNK1/2 and p38 SAP kinase that leads to activation of the transcription factors cJUN and ATF2. These latter factors are in turn required for regulated Map4k4 expression.

Experimental Procedures

Materials- Mouse recombinant Tumor Necrosis Factor Alpha was obtained from Calbiochem (Cat # 654245). Mouse recombinant Interleukin-6, mouse recombinant Interleukin 1β and LPS were obtained from Sigma-Aldrich (Cat# I9646, I5271 and L6529 respectively). Antibodies against IκBα (sc-371), JNK1/2 (sc-7345), phospho cJUN (sc-1694) and phospho-JNK1/2 (sc-6254) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California). Antibodies against ATF2 (9226), phospho-

ATF2 (9221), p38 SAP kinase (9212), and phospho-p38 SAP kinase (9215) were purchased from Cell Signaling Technology Inc. Antibody to stimulate TNF receptor 1 (AF-425-PB) and TNF receptor 2 (ab7369) were purchased from R & D Systems, Inc (Minneapolis, MN) and Abcam, Inc (Cambridge, MA) respectively. TNF receptor 1 neutralizing antibody (MAB430) was also purchased from R & D Systems, Inc. Normal Goat IgG (AB108C) was purchased from R & D Systems, Inc while normal Rat IgG (20005-1) and Hamster IgG (20003) were purchased from Alpha Diagnostics International, Inc (San Antonio, TX). All siRNA was purchased from Dharmacon (Lafayette, CO) and were custom ordered *SMART*pool™ duplexes.

Generation of Map4k4 Antibody- Anti Map4k4 antibody was generated by injecting rabbits with a GST fusion protein corresponding to amino acids 453 to 720 of Map4k4. This hydrophilic region was selected because of predicted high antigenicity and high surface probability. The region was amplified by PCR (forward primer 5'-CCCAGGAATTCGAAGAGGAGAGTGGAGAGGGAACAG-3' and reverse primer 5'-ACGATGCGGCCGCTCCCGCAGGCTTGAGAGACCG-3'), cloned into a pGEX-5 vector and expressed in *E. coli* BL21. Cultures were induced using 1 mM IPTG for 6 hours and then lysed in STE buffer (50 mM Tris pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 ng/ml aprotinin, 5 ng/ml leupeptin, 10 mM PMSF, 1 mM benzamidine, 1 mg/ml lysozyme) followed by the addition of 1% Triton, 2 μg Dnase, 10 mM $MgCL_2$ and 10 mM $MnCL_2$ 30 minutes later. The Map4k4 fusion protein was then incubated with glutathione agarose beads end over end for 1 hour at 4°C. The isolated GST Map4k4 fusion peptide was shipped to Rockland for rabbit injection. 400 μg of protein was injected into two rabbits for the initial immunization followed by three 200 μg injections

for immunological boosts at days 7, 14 and 21 after the initial injection. The IgG fractions were then isolated using Protein A-agarose beads. In order to affinity-purify the anti-Map4k4 antibodies, 1 mg of lysates from HA-Map4K4 expressing COS-1 cells was resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was then cut as strips between 250 KDa and 150 KDa and blocked with 3% BSA TBST for 1 hour. The membrane strip was then incubated overnight with 2 ml of the IgG fraction, washed twice with TBST buffer and twice with PBS. The anti-Map4K4 antibodies were eluted with the addition of 1 ml glycine (100 mM, pH 2.6) to the strips, and incubated for 10 min at room temperature, with occasional vortexing. The eluted antibodies were transferred to a fresh tube, containing 0.1 ml of 1M Tris buffer (pH 8.0) as well 0.1 % BSA and 0.05% azide to bring the final pH to 7.0. Antibodies were stored at -20^0C.

Cell Culture and siRNA Transfection - 3T3-L1 fibroblasts were cultured and differentiated into adipocytes as previously described [297]. For siRNA transfections, cells 4 days post the induction of differentiation were used as previously described [275]. Briefly, 1.125×10^6 cells were electroporated using 6 nmol of siRNA and then plated in 4 wells of a twelve well plate. Cells were recovered in complete DMEM (10% FBS, 50 units/ml Penicillin and 50 μg/ml Streptomycin) and were cultured for 72 hours after the transfection before beginning experiments.

TNFα, IL-1β, IL-6 and LPS stimulation - 3T3-L1 adipocytes, either 7 days post differentiation or 72 hours post siRNA transfection, were washed twice with PBS and treated with the indicated concentrations of TNFα in complete DMEM for the appropriate time intervals. For the cytokine and LPS stimulations, 3T3-L1 adipocytes, 4

days post differentiation induction, were harvested and resuspended in 50 ml of DMEM with per 15 cm plate. The adipocytes were distributed such that 1 ml of the harvested adipocytes were distributed per well of a 12 well plate (Cat # 3515, Corning, Inc, Corning, NY). After 72 hours, the adipocytes were treated with the appropriate concentration of cytokine for the given period of time. In all experiments, cells were washed with ice cold PBS, and harvested on ice as described previously [298]. Protein samples were separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were then analyzed by western blot analysis. Changes in phosphorylation were determined through densitometry using Adobe Photoshop and normalized for loading against the non-phosphorylated kinase or transcription factor, with the exception of cJUN, whose expression increased during the entire stimulation and IκBα whose expression was variable.

TNFR1 and TNFR2 stimulation- 3T3-L1 adipocytes were prepared the same as described for the cytokine treatments. 3T3-L1 adipocytes 7 days post differentiation were treated with the indicated concentrations of antagonistic or agonistic antibodies against TNFR1 or TNFR2 in the presence or absence of TNFα for 24 hours. Antibodies were used at the concentrations recommended by the manufacturers for maximal activity. Antagonistic antibodies were also tested for IL-6 induction at 10 times the concentration recommended and no additional increase in IL-6 mRNA was detected (data not shown). Following treatment the cells were lysed and analyzed the same as for the cytokine treatments.

Isolation of RNA and Semi Quantitative Real Time PCR- RNA isolation was performed according to the Trizol Reagent Protocol (Cat # 15596-018, Invitrogen, Carlsbad CA). Briefly, media was aspirated and the cells were washed once with ice cold PBS. Next, 1 ml of Trizol reagent was added to each well. The concentration and the purity of the RNA was determined by measuring the absorbance at 260/280 nm. To further determine the quality of the RNA 1 μg of total RNA was run on a 1% agarose gel and the quality of the 28S and 18S ribosomal bands were inspected visually. cDNA was synthesized using 1 μg of RNA and the iScript cDNA Sythesis Kit (Cat # 170-8891) from Bio Rad (Hercules, CA). The cDNA was synthesized according to the protocol provided by the manufacturer in a 20 μl reaction volume. For real time PCR, 1 μl of the synthesized cDNA was loaded into one well of a 96 well plate for detection of a specific target gene. Primers used are listed in Supplementary Figure 2.1 and were designed with Primer Bank [299]. HPRT was used as an internal loading control as its expression did not change over a 24 hour period with the addition of TNFα and the silencing of the genes used in this study. 10 pmol of forward and reverse primer along with 12.5 μl of the iQ SYBR Green Supermix (Bio Rad) was added to each well along with DNase/RNase free water for a final volume of 25 μl Samples were run on the MyIQ Realtime PCR System (Bio Rad). Relative gene expression was determined using the delta CT method [300].

Statistics- The distributional characteristics of the outcomes were evaluated by both a visual inspection of histograms and the Kolmogorov-Smirnoz test performed on model residuals. Transformations by natural logarithms was used in some cases to better approximate a normal distribution and to stabilize variances. The observed effects were

evaluated by either one-way or multifactorial analysis of variance (ANOVA). In the presence of significant main and/or interaction effects pairwise comparisons were made using the Tukey (HSD) multiple comparisons procedure with the exception of Figure 2.3 where the Tukey-Kramer method was used due to unequal sample sizes between the groups. Computations were performed using SAS Statistical software package. The data are presented as means +/- SEM.

Results

TNFα signaling upregulates the expression of Map4k4 protein and mRNA - To better characterize the TNFα-mediated increase in Map4k4 mRNA expression, we examined the dose-response relationship and time course of TNFα action in 3T3-L1 adipocytes (Fig. 2.1A and 2.1B). The fully differentiated adipocytes were treated with various concentrations of TNFα, ranging from 0.1 ng/ml to 100 ng/ml for 24 h. The cells were then lysed, RNA was isolated, and the Map4k4 mRNA levels were compared using quantitative real time PCR. A maximal increase in Map4k4 mRNA in response to 5-10 ng/ml TNFα was observed that reached about 3 fold (Fig. 2.1A). This concentration of TNFα corresponds to previously reported concentrations of TNFα which caused maximal impairment of insulin signaling in 3T3-L1 adipocytes [113]. A time course analysis of the increase in Map4k4 mRNA following 50 ng/ml TNFα showed a 2 fold increase in Map4k4 mRNA at two hours which increased by 48 hours to a maximal stimulation of over 4 fold (Fig. 2.1B). To determine whether the increase in Map4k4 mRNA resulted in a corresponding increase in protein, adipocytes were treated with 50 ng/ml TNFα for 24 hours, lysed and immuno-blotted with an antibody we raised in

rabbits. At 24 hours after the addition of TNFα, a 2.5 fold increase in Map4k4 protein levels was observed (Fig. 2.1C and 2.1D). Thus, treatment with TNFα significantly increases Map4k4 expression in 3T3-L1 adipocytes.

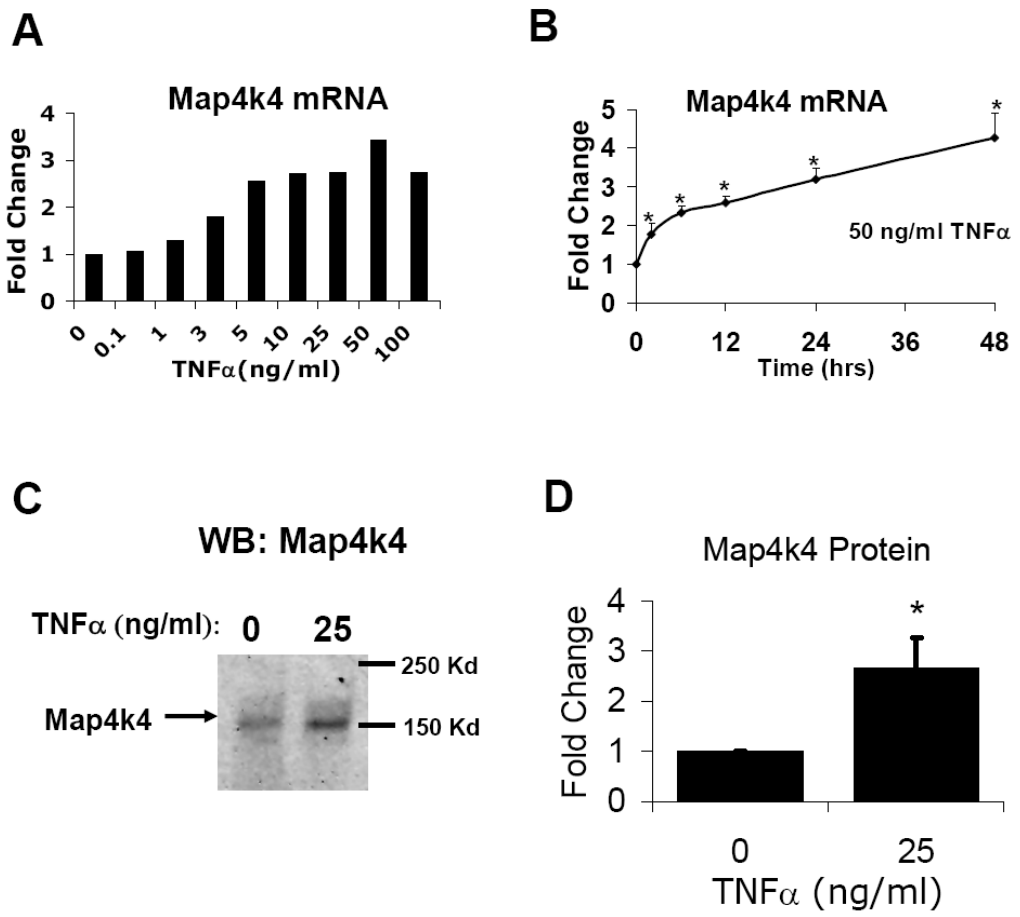


Figure 2.1. Treatment of 3T3-L1 Adipocytes with TNFα causes increased Map4k4 expression that is both dose and time dependent. **A.** A representative experiment demonstrating the dose dependent relationship of the TNFα increase in Map4k4 mRNA. 3T3-L1 adipocytes were treated with the indicated concentrations of TNFα for a 24 hour period. Total RNA was harvested and Map4k4 mRNA was quantified using real time PCR. **B.** Time course of the increase in Map4k4 mRNA upon treatment with 50 ng/ml TNFα. $p<.05$ (*) and $p<.01$ (**) when compared to 0 hours by ANOVA and Tukey's HSD test. (n=3) **C.** 24 hr TNFα treatment increases Map4k4 protein expression. 3T3-L1 adipocytes after 7 days of differentiation were treated with TNFα for 24 hours, lysed and analyzed by western blot **D.** Densitometry analysis of 3 western blot experiments of 3T3-L1 adipocytes treated with TNFα. * = $p<.05$ when compared to samples without TNFα treatment by students t-test. Results are the mean ± SEM. The data in Figure 1A was obtained by Xiaoqing Tang. The GST construct to produce MAP4K4 antibody was made by Anil Chawla and purified by Kalyani Guntur, Andrea Hubbard and me. I produced and analyzed all other data.

Map4k4 has been proposed to act in a cascade that stimulates JNK1/2 activity in response to TNFα through a MKK4- and MKK7-dependent mechanism [183]. To determine if TNFα increases the expression of Map4k4 mRNA selectively among the Map kinase family, we treated 3T3-L1 adipocytes with various concentrations of TNFα over a 24 hour period, and then measured Map4k4, MKK4, MKK7, JNK1, JNK2, ERK1, and ERK2 protein kinase mRNA levels using quantitative real time PCR. Map4k4 mRNA levels increased 1.5, 2.6 ($p < .01$) and 3.4 fold ($p < .01$) with 1, 5 and 25 ng/ml TNFα, respectively (Fig. 2.2). In contrast, the expression of MKK4, MKK7, ERK2, JNK1 and JNK2 was unperturbed by TNFα signaling. ERK1 mRNA expression was found to increase only very slightly 1.2, 1.7 ($p < .01$) and 1.4 fold ($p < .05$) at 1, 5 and 25 ng/ml TNFα. Furthermore, in other studies p38 SAP kinase protein expression did not change during treatment with 25 ng/ml TNFα (see Fig. 2.4B). Taken together, these results suggest that the increase in Map4k4 expression following TNFα treatment is unique among the Map kinases we tested in its high responsiveness to TNFα signaling.

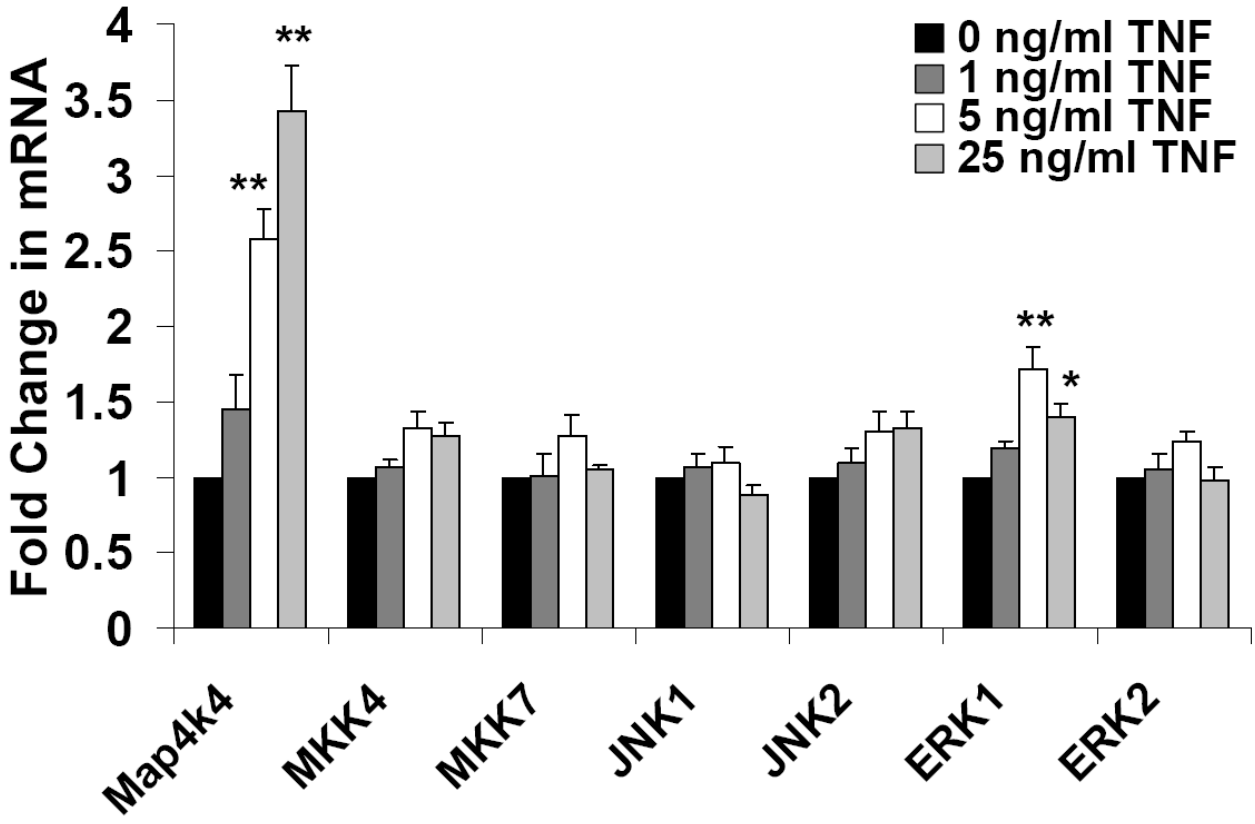


Figure 2.2. Map4k4 mRNA expression in comparison to expression of other Map kinases in response to TNFα. 3T3-L1 adipocytes were treated with indicated concentrations of TNFα for 24 hours. Total RNA was harvested and mRNA expression levels of the indicated Map kinases were quantified using real time PCR. $p < .05$ (*) while $p < .01$(**) when compared to the basal level for the given map kinase by ANOVA and Tukey's HSD test. For Map4k4, JNK1/2 and ERK1/2 n=5 and for MKK4 and MKK7 n=3. Results are the mean ± SEM. The data for this figure was produced by Adilson Guilherme and me.

Map4k4 expression is specifically augmented by TNFα and unresponsive to IL-1β, IL-6 and LPS. TNFα and other cytokines, such as IL-1β and LPS, initiate similar intracellular signaling networks that activate p38 SAP kinase and JNK1/2 along with the transcriptional regulator NFκB (Fig. 2.3A) (for reviews see [301-303]). In addition, TNFα treatment of cultured and primary adipocytes increases expression and secretion of other cytokines such as IL-6, which signals through the JAK/STAT pathway (Fig. 2.3A) [122,123]. Thus, the increase in Map4k4 expression observed in response to TNFα may result from the secretion and actions of these secondary cytokines rather than TNFα itself. Indeed, IL-6 expression was shown in our studies to be stimulated over 40 fold by TNFα in cultured adipocytes (Fig. 2.3C, top panel). To test whether these other cytokines also increase Map4k4 expression, we treated 3T3-L1 adipocytes with TNFα (50 ng/ml), IL-1β (100 ng/ml), LPS (100 ng/ml) or IL-6 (50 ng/ml) for 24 hours. The concentrations of these agents tested were shown in our preliminary studies to be well in excess of the concentrations necessary for their maximal effects on expression of other genes (data not shown). In all of these experiments, only TNFα was able to increase Map4k4 expression under these conditions (Fig. 2.3B). That IL-1β, LPS and IL-6 do initiate other potent biological effects under these same conditions is indicated in Figure 2.3C. Thus, IL-1β and LPS stimulate IL-6 expression about 10 fold, while IL-6 stimulates Socs3 mRNA several fold (Fig. 2.3C). Taken together, these results suggest that TNFα increases Map4k4 expression in cultured adipocytes through a signaling mechanism unique to TNFα, at least compared to the other cytokines tested in these experiments.

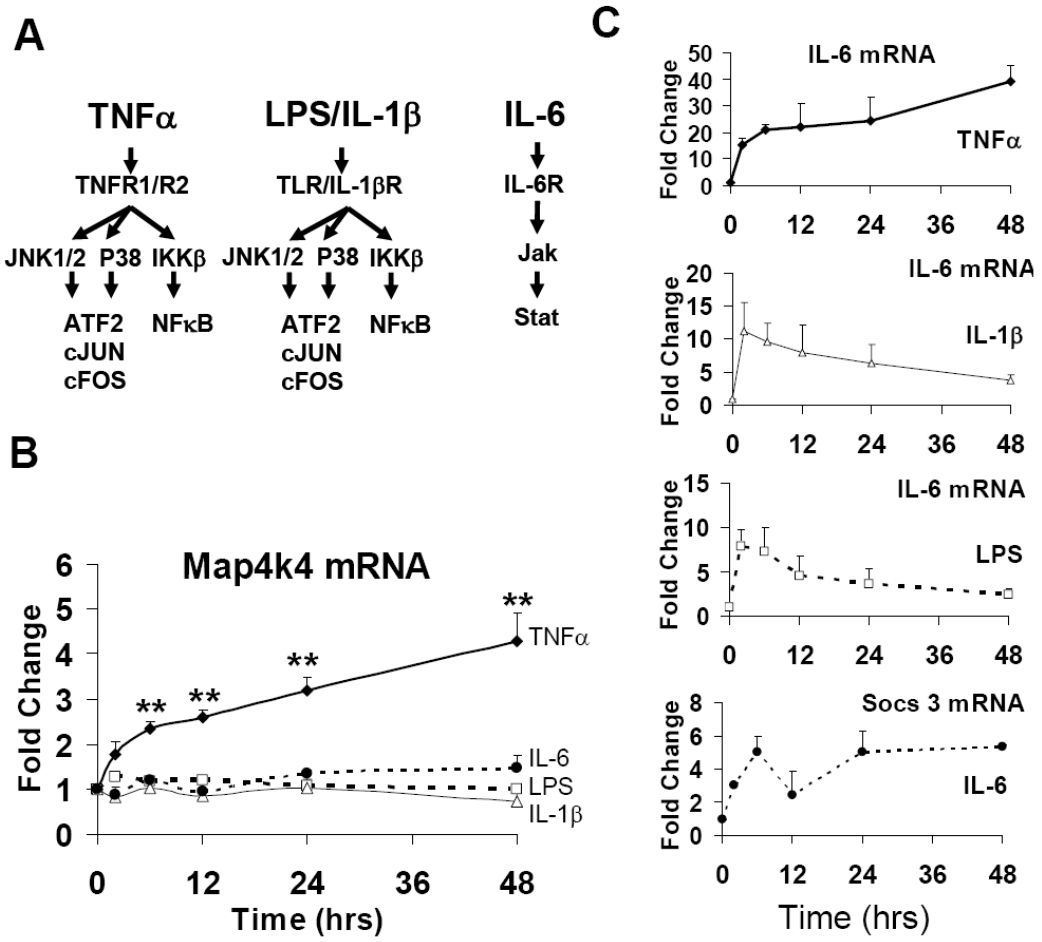


Figure 2.3. Map4k4 mRNA is increased by TNFα, but not by IL-1β, LPS or IL-6. **A**. A representative schematic of intracellular signaling pathways activated by TNFα, IL-1β, LPS and IL-6. Briefly, TNFα, IL-1β and LPS bind the TNFα, IL-1 and toll-like receptors (TLR) respectively to initiate an intracellular response culminating with the activation of many similar transcription factors including AP-1 and NFκB. However, IL-6 activates different intracellular pathways such as the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathways. 3T3-L1 adipocytes 7 days post differentiation induction were treated with the appropriate stimuli for the indicated period of time. **B**. Time course of the Map4k4 mRNA increase in response to TNFα (50 ng/ml, ♦), IL-1β (100 ng/ml, Δ), LPS (2 μg/ml, □) and IL-6 (50 ng/ml , ●) in 3T3-L1 adipocytes. $p < .01$(**) for TNFα treatment against all of the other individual cytokine treatments analyzed by two-way ANOVA with Tukey-Kramer post test. **C**. Time course for IL-6 or SOCS3 mRNA induction in response to TNFα, IL-1β and LPS or IL-6 respectively. Cytokine stimulations were repeated 4 times for TNFα, IL-1β and IL-6 and 3 times for LPS. Results are the mean ± SEM. All data was produced and analyzed by me.

***Signaling by TNFα, but not IL-1β, causes prolonged phosphorylation of JNK1/2 protein kinases, p38 SAP kinase, cJUN and ATF2*.** Since TNFα and IL-1β are thought to activate identical intracellular signaling pathways in 3T3-L1 adipocytes, we sought to compare the activation of these pathways by TNFα versus IL-1β utilizing phospho-specific antibodies targeting the downstream protein kinases and transcription factors. Cultured adipocytes were treated with TNFα (50 ng/ml) and IL-1β (100 ng/ml) for various periods of time over a 24 hour period. To examine NFκB activation in parallel, we utilized antibodies recognizing total IκBα, which is rapidly degraded to initiate NFκB-mediated transcription. Interestingly, both TNFα and IL-1β caused the rapid loss of the NFκB inhibitor protein, IκBα suggesting that both cytokines activated NFκB in a similar manner (Fig. 2.4A). On the other hand, the activation of p38 SAP kinase was both more dramatic and more sustained in response to TNFα compared to IL-1β treatment (Fig. 2.4B). Specifically, TNFα caused a 70 fold increase in p38 SAP kinase phosphorylation at 10 minutes, while IL-1β caused only a 20 fold increase in p38 SAP kinase phosphorylation at this early time point. The TNFα-induced phosphorylation did subside somewhat, but was maintained at levels near 20 fold over basal for over 2 hours and was dramatically higher than basal for over 6 hours (Fig. 2.4B). The effect of IL-1β in contrast, returned to basal levels within 1 hour. This difference in p38 SAP kinase phosphorylation caused by TNFα versus IL-1β correlated well with p38 SAP kinase activation as the phosphorylation of its substrate ATF2 by TNFα, but not by IL-1β, was also sustained over the later time points (Fig. 2.5A).

Similar to the data obtained for p38 SAP kinase activation, both JNK1 and JNK2 protein kinases exhibited large increases in phosphorylation during the first few minutes of treatment with either TNFα or IL-1β. JNK1 and JNK2 are both expressed as isoforms with molecular weights of 46 and 55 kDal (Fig. 2.4C and 2.4D). TNFα-induced phosphorylations of the JNK 46 kDal and JNK 55 kDal species was maintained at increases of about 20-fold and 5-fold, respectively, for a full 24 hours, while IL-1β induced phosphorylations in JNK subsided to near basal levels within the first hour of stimulation (Fig. 2.4C and 2.4D). The phosphorylations of JNK1/2 correlated well with the phosphorylation of their substrate transcription factors ATF2 (Fig. 2.5A) and cJUN (Fig. 2.5B), which showed similar sustained increases in phosphorylation in response to TNFα but not to IL-1β. Furthermore, the increase in cJUN phosphorylation was accompanied by a substantial increase in total cJUN protein (Fig 2.5C). This is consistent with previous work identifying cJUN as a positive regulator of its own transcription [304,305]. Overall, these data suggest that although the signaling pathways activated by TNFα and IL-1β are similar, the kinetics of activation of the JNK1/2 and p38 SAP kinase are quite different. Furthermore, the sustained phosphorylation of JNK1/2, p38 SAP kinase, cJUN and ATF2 in response to TNFα correlates with its action to increase Map4k4 mRNA.

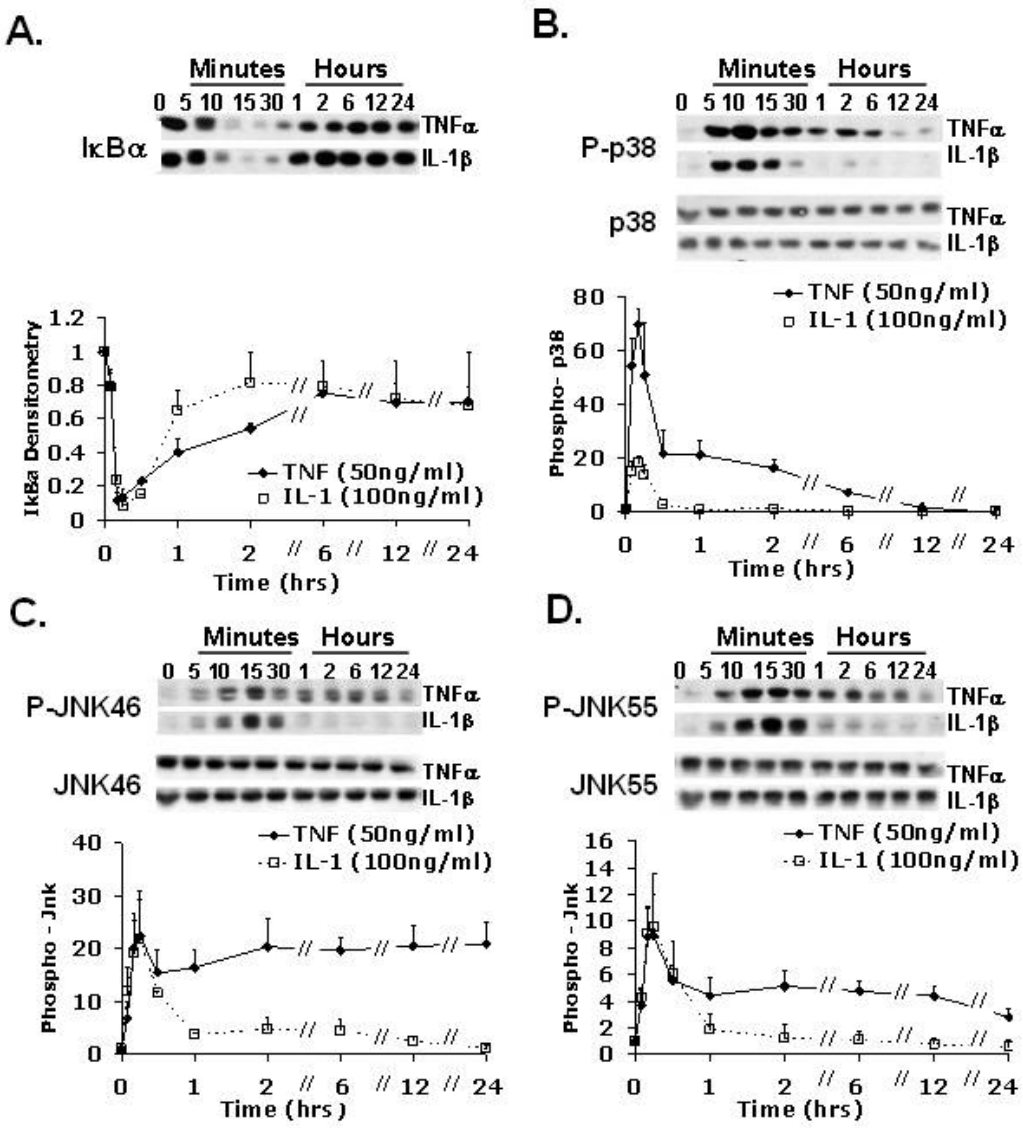


Figure 2.4. TNFα, but not IL-1β, causes enhanced phosphorylation of p38 SAP kinase and JNK1/2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes 7 days post differentiation induction were treated with either 50 ng/ml TNFα or 100 ng/ml IL-1β. After the indicated period of time, cells were harvested and lysates were examined by western blot and densitometry analysis for IκBα (**A)** and phospho-p38 SAP kinase (**B**). Phosphorylation of JNK1 and JNK2 which are both comprised of 46 and 55 kDal isoforms were analyzed in **C** (JNK46) and **D** (JNK55) respectively. Densitometry is representative of three independent experiments. Results are the mean ± SEM. All data was produced and analyzed by Adilson Guilherme and me.

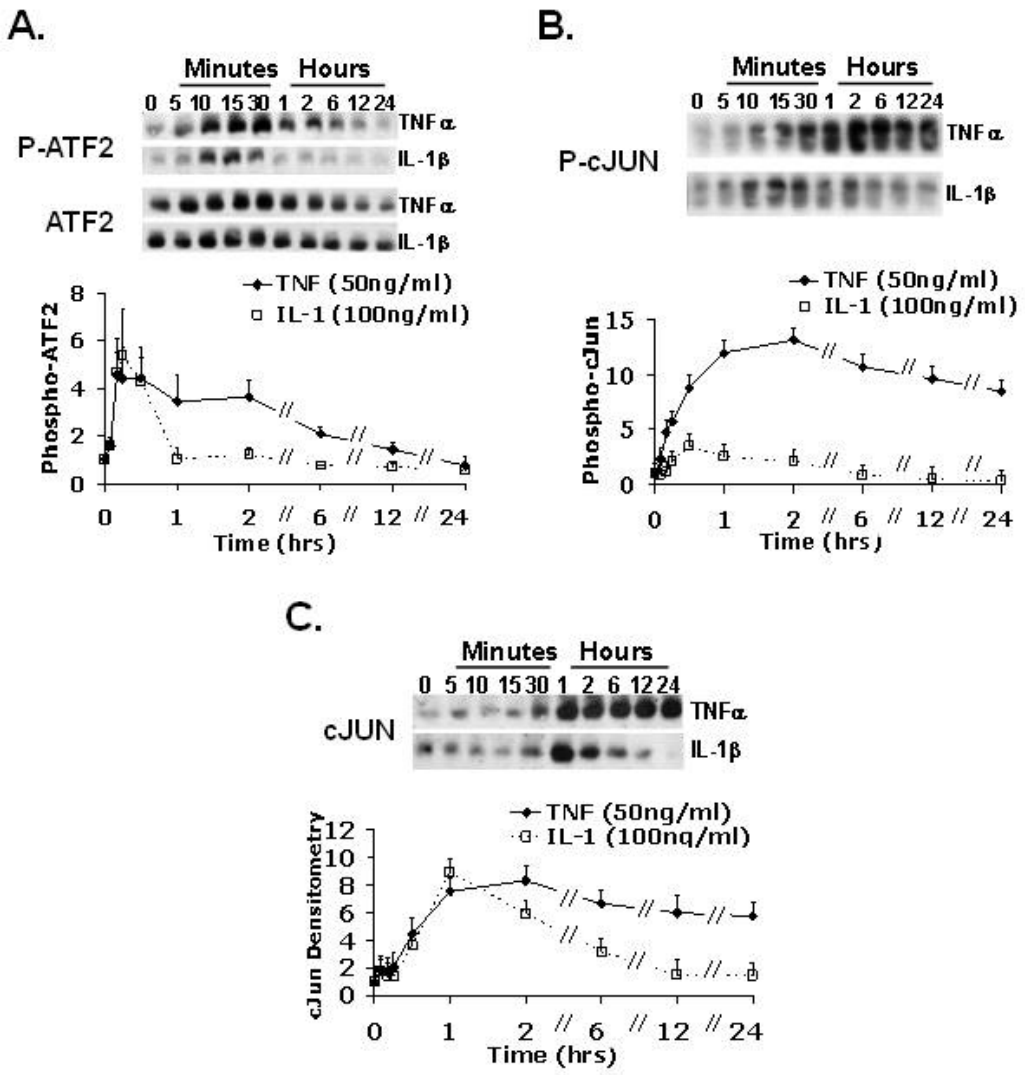


Figure 2.5. TNFα, but not IL-1β, causes enhanced phosphorylation of cJUN and ATF2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes 7 days post differentiation induction were treated with either 50 ng/ml TNFα or 100 ng/ml IL-1β for the indicated period of time and then lysed. Cell lysates were examined by western blot and densitometry analysis for phospho-ATF2 (**A),** phospho-cJUN (**B**), and total cJun (**C**). Densitometry is representative of three independent experiments. Results are the mean ± SEM. All data was produced and analyzed by Adilson Guilherme and me.

Depletion of cJUN and ATF2 attenuates the TNFα mediated increase in Map4k4 mRNA expression. ATF2 and cJUN are among numerous transcription factors that are activated in response to TNFα treatment [301,303], and our data described above suggested that Map4k4, cJUN and ATF2 are all differentially regulated by TNFα versus IL-1β. Both of these transcription factors are only transiently activated following stimulation with IL-1β, while TNFα provokes a robust and sustained phosphorylation of cJUN and ATF2 in 3T3-L1 adipocytes (Figs. 2.4 and 2.5). Thus, it is plausible that the sustained activation of these transcription factors by TNFα is required to cause the increase in Map4k4 expression (Figs. 2.1-3). To address the role of cJUN and ATF2 in TNFα action on the expression of Map4k4, we depleted cJUN and ATF2 in 3T3-L1 adipocytes using siRNA and then treated the cells with various concentrations of TNFα over a 24 hour period. Silencing either cJUN or ATF2 caused a dramatic decrease in Map4k4 expression in the presence of 1, 5 and 25 ng/ml TNFα (Fig. 2.6A). The decrease was highly statistically significant ($p<.01$) for 5 and 25 ng/ml TNFα upon silencing cJUN, ATF2 or both molecules in combination. Additionally, when silencing both transcription factors simultaneously, we observed a decrease in the augmentation of Map4k4 mRNA expression in response to 1 ng/ml TNFα ($p<.05$). Validation of the siRNA-based decreases in cJUN and ATF2 mRNA and protein (not shown) revealed reductions of approximately 50% (Fig. 2.6C and D). Interestingly, silencing of cJUN and ATF2 did not significantly impair the up-regulation of IL-6 mRNA (Fig. 2.6B). This is consistent with previous work, which identified Fra-1 and JunD but not cJUN or ATF2 as interacting with the AP-1 binding sequence in the IL-6 promoter in the presence and

absence of TNFα stimulation [306,307]. Altogether, these results combined with the observed sustained activation of cJUN and ATF2 in response to TNFα (Fig. 2.5A and 2.5B), suggest that cJUN and ATF2 are required for TNFα-enhanced levels of Map4k4.

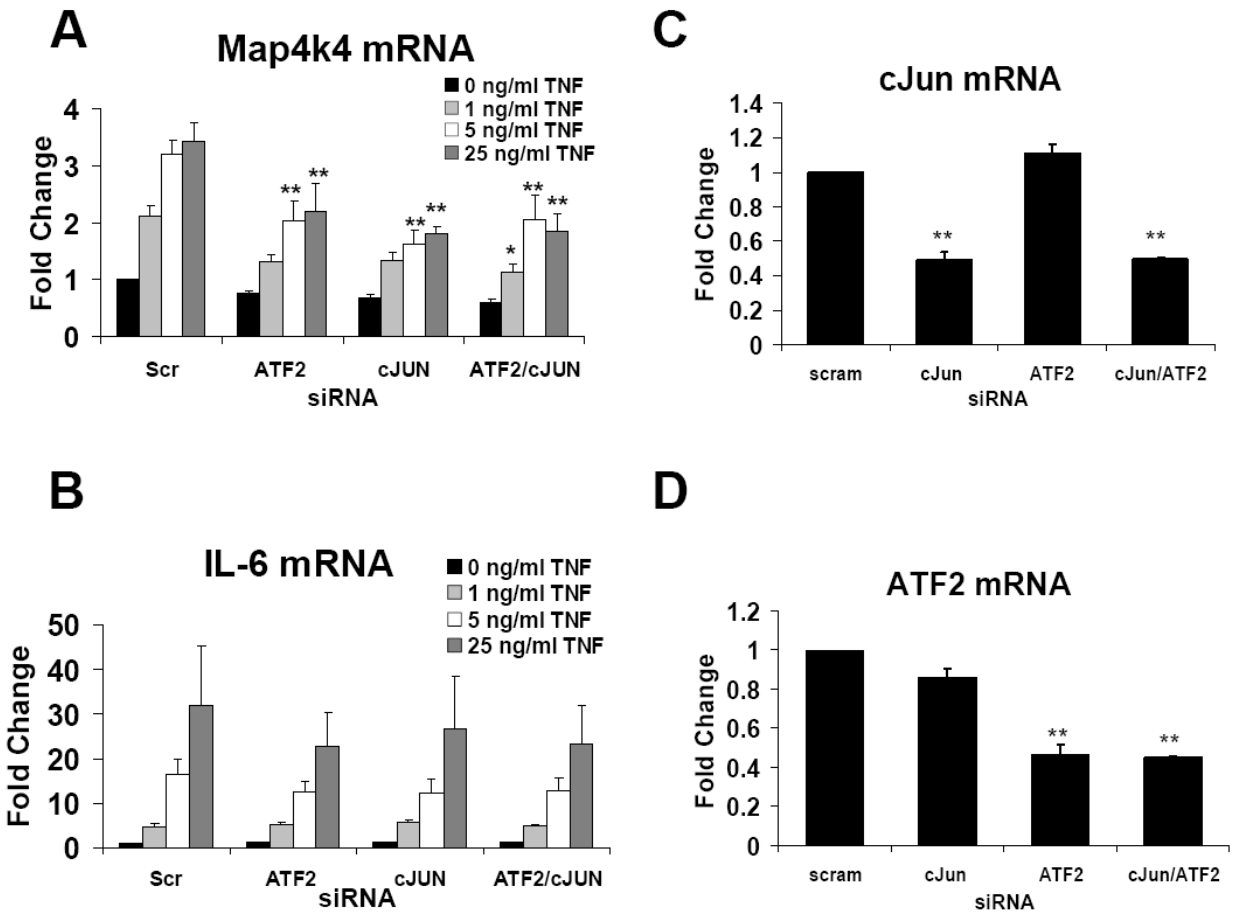


Figure 2.6. Silencing of cJUN and ATF2 attenuates the TNFα-mediated increase in Map4k4 expression. 3T3-L1 adipocytes 4 days post differentiation induction were transfected with 6 nmol of either scrambled, ATF2, cJUN or cJUN plus ATF2 siRNA. 48 hours later the cells were treated with the indicated concentrations of TNFα for a 24 hour period. Total RNA was harvested and analyzed by quantitative real time PCR for Map4k4 mRNA expression (**A.**) and IL-6 mRNA expression (**B.**). $p < .05$ (*) while $p < .01$ (**) against the corresponding TNFα treatment in the scrambled siRNA sample by two-way ANOVA with Tukey's HSD test. To validate the knockdown cJUN (**C.**) and ATF2 (**D.**) mRNA expression was assessed. $p < .05$ (*) and $p < .01$ (**) against scrambled as analyzed by ANOVA with Tukey's HSD test. (n=4). Results are the mean ± SEM. All data was produced and analyzed by me.

TNFR1 stimulation increases Map4k4 mRNA expression. TNFα exerts its effects through two distinct receptors, denoted type 1 (TNFR1) and type 2 receptors (TNFR2) [303]. The roles of these receptors in the inhibition of insulin action, lipolysis and the negative regulation of adipocyte-specific genes has been well characterized [124,296,308], and mostly attributed to TNFR1. To determine whether TNFR1 or TNFR2 is responsible for increasing Map4k4 expression specifically, we first utilized antibody preparations with specific agonistic properties for TNFR1 and TNFR2 as well as antagonistic properties for TNFR1. In agreement with our previous experiments (Fig. 2.1A + B, 2.2 and2. 3B), 24 h of TNFα treatment significantly increased Map4k4 mRNA expression approximately 3 fold in 3T3-L1 adipocytes ($p<.01$) (Fig. 2.7A). Incubation of the cells with TNFR1 antagonistic antibodies almost completely blocked this effect of TNFα, while TNFR1 agonistic antibodies strongly mimicked the effect ($p<.01$) (Fig. 2.7A). Interestingly, agonistic antibodies against TNFR2 failed to increase Map4k4 mRNA expression (Fig. 2.7A). Non-immune IgG antibodies from the appropriate sources had no effect on either Map4k4 expression in TNFα stimulated or unstimulated conditions (data not shown). These results suggest that TNFα-stimulated Map4k4 expression is mediated through TNFR1, not TNFR2.

To assess whether the antibodies were functioning properly we measured the changes in IL-6 mRNA expression, a cytokine highly upregulated in adipocytes following TNFα stimulation [122]. In agreement with previous studies (Fig 2.3C and 2.6B), TNFα caused a 20 fold increase in IL-6 mRNA expression ($p<.01$) (Fig. 2.7B). Blocking the actions of TNFR1 with antagonistic antibodies significantly blunted the effect of TNFα by

approximately 75%. However, adipocytes treated with the TNFR1 antagonistic antibodies and TNFα still showed a 4.5 fold increase in IL-6 mRNA, which presumably was due to the activity of TNFR2 (Fig. 2.7B). In agreement, the TNFR2 agonistic antibodies increased IL-6 mRNA expression 4 fold. Taken together, these results suggest that the TNFR1 antagonistic antibodies are potent inhibitors of TNFR1 function under the conditions of our experiments (Fig. 2.7B). Furthermore, TNFR1 agonistic antibodies increased IL-6 mRNA approximately 13 fold ($p<.01$), suggesting that unlike the increase in Map4k4 mRNA, the increase in IL-6 mRNA requires the actions of both receptors.

We also addressed the role of TNFR1 and TNFR2 in increasing Map4k4 mRNA following TNFα treatment by using siRNA to deplete the expression of these receptors in 3T3-L1 adipocytes. In agreement with the results using antagonistic and agonistic TNFα receptor antibodies, silencing of TNFR1, but not TNFR2, significantly blunted the TNFα increase in Map4k4 mRNA (Fig. 2.7C). Silencing was confirmed by assessing mRNA levels of TNFR2 and TNFR1, which showed approximately 70% and 60% depletions, respectively (Fig. 2.7D). Altogether, this data suggests that signaling through TNFR1 increases Map4k4 expression following TNFα treatment in cultured adipocytes.

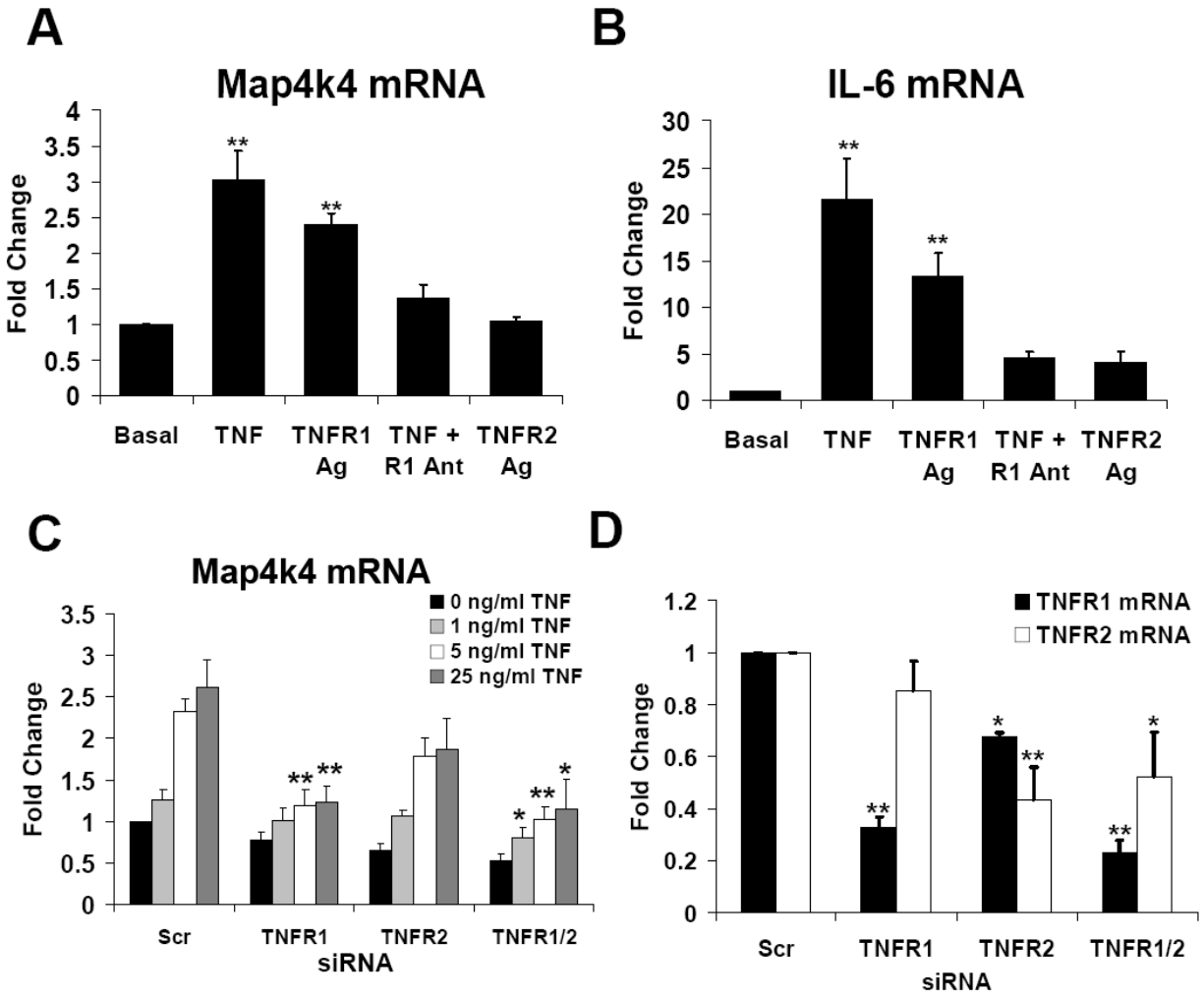


Figure 2.7. TNFα increases Map4k4 expression through TNFR1 but not TNFR2. 3T3-L1 adipocytes 7 days post differentiation induction were treated as follows: 50 ng/ml TNFα, 5 ng/ml TNFR1 agonistic antibodies plus 50 ng/ml TNFα, 5 ng/ml TNFR1 antagonistic antibodies and 50 ng/ml TNFR2 antagonistic antibodies. Total RNA was isolated after 24 hours of treatment and analyzed by quantitative real time PCR for Map4k4 mRNA (n=4) (**A.**) and for IL-6 mRNA (n=4) (**B.**). $p < .01$ (**) against basal as analyzed by ANOVA with Tukey's HSD test. 3T3-L1 adipocytes 4 days post differentiation induction were transfected with 6 nmol of either scrambled, TNFR1, TNFR2 or TNFR1 and TNFR2 siRNA. 48 hours later the cells were treated with the indicated concentrations of TNFα for a 24 hour period. Total RNA was isolated and analyzed by quantitative real time PCR for Map4k4 mRNA (n=4) (**C.**). $p < .05$ (*) while $p < .01$ (**) against the corresponding TNFα treatment in the scrambled siRNA sample by two-way ANOVA with Tukey's HSD test. The efficiency of siRNA mediated silencing was determined by quantitative real time PCR for TNFR1 and TNFR2 mRNA (**D.**). $p < .05$ (*) and $p < .01$ (**) against scrambled as analyzed by ANOVA with Tukey's HSD test. Results are the mean ± SEM. All data was produced and analyzed by me.

To determine whether unique signaling pathways are activated by TNFR1, which may account for the increase in Map4k4 mRNA expression, downstream signaling elements from TNFR1 and TNFR2 were dissected using agonistic antibodies. 3T3-L1 adipocytes were treated with TNFα, TNFR1 or TNFR2 agonistic antibodies for various periods of time over 12 hours, and activation of JNK1/2, p38 SAP kinase, cJUN and ATF2 was assessed using phospho-specific antibodies. Additionally, the disappearance of IκBα was used to estimate NFκB activation. Stimulation of TNFR1 resulted in a similar increase in phosphorylation of JNK1/2, p38 SAP kinase, cJUN and ATF2 as well as a similar loss of IκBα and increase in total cJUN protein when compared to TNFα treatment (Fig. 2.8A-D and Fig. 2.9A-C). TNFR2 stimulation did not activate most intracellular signaling pathways tested, although we did observe a slight decrease in IκBα at the later time points, suggesting a possible slight activation of NFκB (Fig. 2.8A). Additionally, p38 SAP kinase (Fig. 2.8B) and ATF2 (Fig. 2.9A) phosphorylation increased to 5 and 2 fold over basal, respectively, following TNFR2 stimulation. Thus, these data are similar to our previous results observed regarding the differences between IL-1β and TNFα signaling (Figs. 2.4 and 2.5), in which cJUN and ATF2, but not NFκB activation correlates with the increase in Map4k4 mRNA expression.

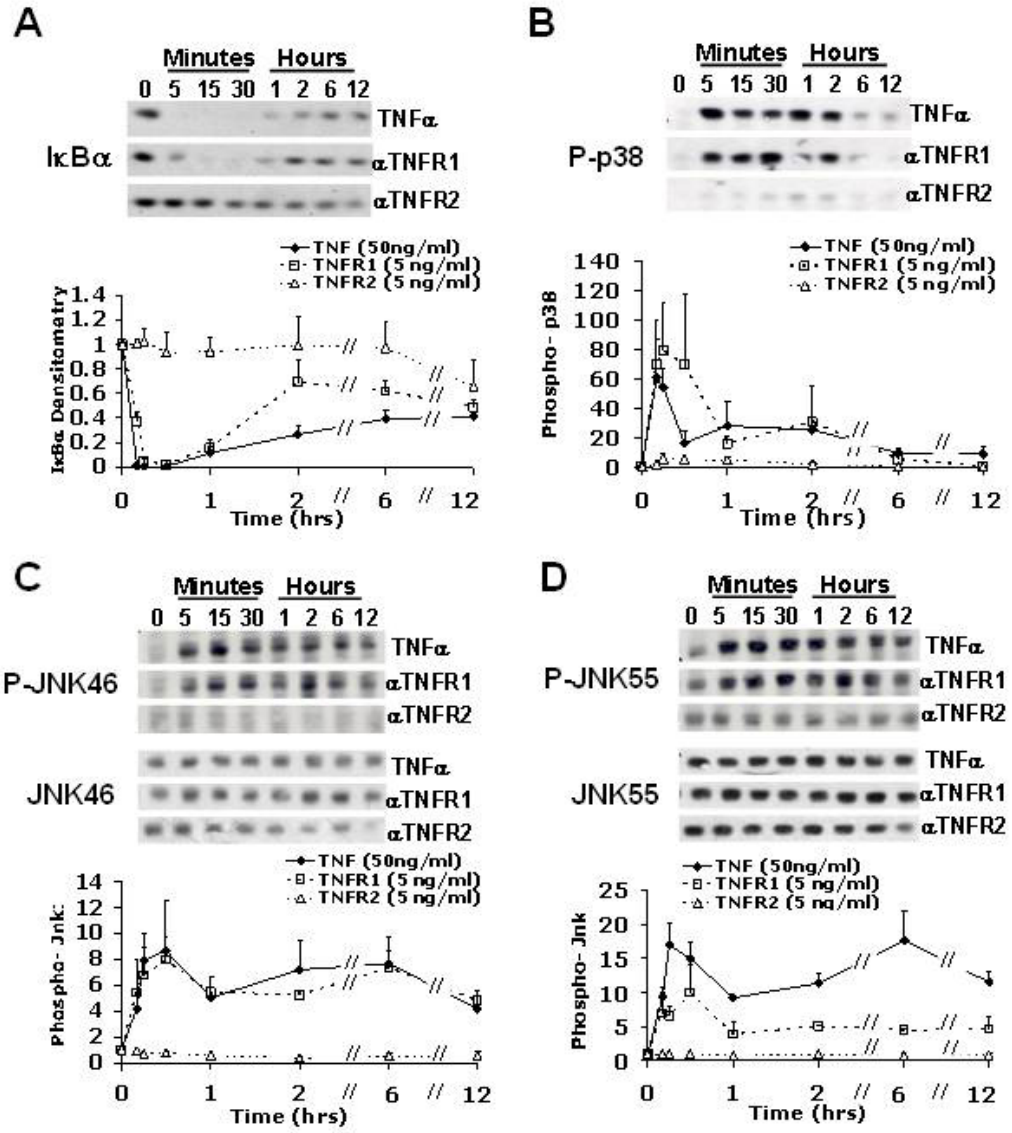


Figure 2.8. TNFR1 activation, but not TNFR2 activation, increases JNK1/2 and p38 SAP kinase phosphorylation. 3T3-L1 adipocytes 7 days post differentiation induction were treated with either 50 ng/ml TNFα, 5 ng/ml TNFR1 agonistic antibodies or 50 ng/ml TNFR2 agonistic antibodies. Total protein was harvested and evaluated by western blot analysis for IκBα (**A.**) phospho-p38 SAP kinase (**B.),** phospho-JNK46 (**C.**) and phospho-JNK55 (**D.**). Densitometry is representative of three independent experiments except for phospho-p38 which was repeated twice. Results are the mean ± SEM. All data was produced and analyzed by me.

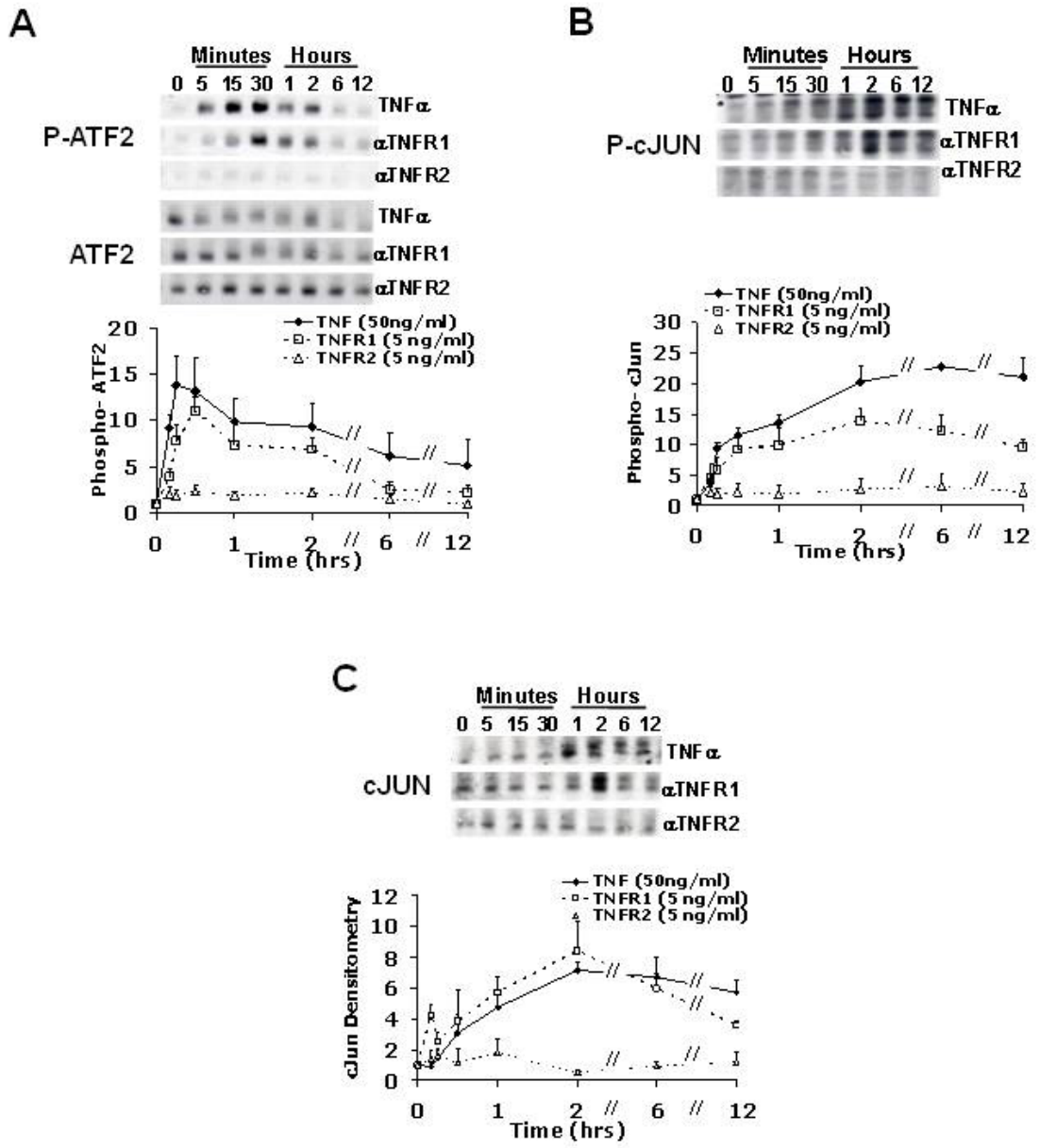


Figure 2.9. TNFR1, but not TNFR2 activation increases the phosphorylation of cJUN and ATF2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes 7 days post differentiation induction were treated with either 50 ng/ml TNFα, 5 ng/ml TNFR1 agonistic antibody or 50 ng/ml TNFR2 agonistic antibody for the indicated period of time and then lysed. Cell lysates were examined by western blot and densitometry analysis for phospho-ATF2 (**A),** phospho- cJUN (**B**) and total cJUN (**C**). Densitometry is representative of three independent experiments except for total cJun which was repeated twice. Results are the mean ± SEM. All data was produced and analyzed by me.

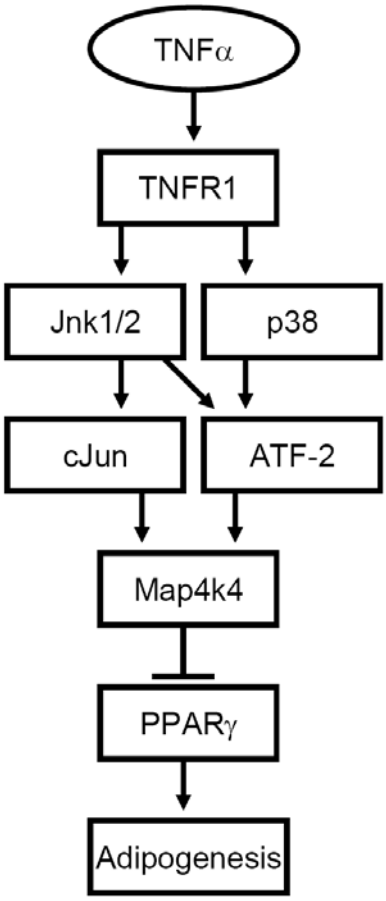


Figure 2.10. Model for the increase in Map4k4 expression via TNFα signaling. Our data are consistent with the following hypothesis: Treatment of 3T3-L1 adipocytes with TNFα stimulates TNFR1 and causes enhanced activation of JNK1/2 and p38 SAP kinase. In turn, activated JNK1/2 and p38 SAP kinase cause increased phosphorylation and thus activation of cJUN and ATF2. Increased activation of cJUN and ATF2 leads to increased Map4k4 transcription, thus increasing Map4k4 expression. This increase in Map4k4 expression then negatively regulates PPARγ expression and adipogenesis in 3T3-L1 adipocytes.

Primer	Sequence
HPRT-F	TCAGTCAACGGGGGACATAAA
HPRT-R	GGGGCTGTACTGCTTAACCAG
Map4K4-F	CATCTCCAGGGAAATCCTCAGG
Map4K4-R	TTCTGTAGTCGTAAGTGGCGTCTG
MKK4-F	AATCGACAGCACGGTTTACTC
MKK4-R	TGAAATCCCAGTGTTGTTCAGG
MKK7-F	TGGCCGCCTTGTTGACTCCAAA
MKK7-R	TTGCCAGCTCCACCAGTGAGAT
JNK1-F	TGTGTGCAGCTTATGATGCCA
JNK1-R	GCTTAGCATGGGTCTGATTCTGA
JNK2-F	CGGACTCAACTTTCACTGTTCT
JNK2-R	AGCACAAACAATTCCTTGGGC
ERK1-F	ACCACATTCTAGGTATCTTGGGT
ERK1-R	GATGCGCTTGTTTGGGTTGAA
ERK2-F	CAGACATGAGAACATCATTGGCA
ERK2-R	TAAAGGTCCGTCTCCATGAGG
IL-6-F	TAGTCCTTCCTACCCCAATTTCC
IL-6-R	TGGGTCCTTAGCCACTCCTTC
SOCS3-F	ATGGTCACCCACAGCAAGTTT
SOCS3-R	TCCAGTAGAATCCGCTCTCCT
cJUN-F	CCTTCTACGACGATGCCCTC
cJUN-R	GGTTCAAGGTCATGCTCTGTTT
ATF2-F	AAGTCTGGCTATCATACTGCTGA
ATF2-R	GCCATGACAATCTGTGAAAGTGC
TNFR2-F	ACACCCTACAAACCGGAACC
TNFR2-R	AGCCTTCCTGTCATAGTATTCCT
TNFR1-F	GGGCACCTTTACGGCTTCC
TNFR1-R	GGTTCTCCTTACAGCCACACA

Supplementary Table 2.1 Primer sequences used in this study

Discussion

TNFα promotes cellular inflammatory responses by altering gene expression through NFκB and AP-1 signaling [301,303], and appears to play significant roles in insulin resistance in obese mice [111,293] and humans [110,294,295]. The protein kinase Map4k4 is a proximal element in the TNFα signaling cascade [183,289] and may mediate in part TNFα actions on PPARγ and GLUT4 that impair insulin responsiveness in adipocytes [275]. Furthermore, TNFα appears to utilize Map4k4 to cause insulin resistance in muscle, as siRNA depletion of Map4k4 in muscle tissue from diabetic patients completely restored insulin sensitivity [289]. In this study, we document that TNFα signaling increases the expression of Map4k4 in 3T3-L1 adipocytes (Fig. 2.1), potentially amplifying its own acute signaling pathway. Our findings lead to the model summarized in Figure 2.10, in which TNFα activates TNFR1, causing a robust and potentiated activation of JNK1/2 and p38 SAP kinase (Fig. 2.4 and 2.8). According to this model, activation of JNK1/2 and p38 SAP kinase cause increased phosphorylation of ATF2 and cJUN along with an increase in total cJUN protein (Fig. 2.5 and 2.9). An important finding of this study was that cJUN and ATF2 were required for optimal stimulation of Map4k4 expression by TNFα (Fig. 2.6). Taken together, these results provide mechanistic insight into TNFα signaling, and indicate that in addition to acutely activating Map4k4 protein kinase activity [183], the cytokine enhances the abundance of this kinase through transcriptional regulation. As previously shown, siRNA depletion of Map4k4 partially prevents the TNFα depletion of GLUT4 and adipogenesis in cultured adipocytes [275]. Hence, the

increased activity (data not shown here) and the increased amount of Map4k4 induced by TNFα would be expected to provide a potent stimulus to decrease adipogenesis and inhibit glucose uptake.

The kinetics of phosphorylation of JNK1/2, p38 SAP kinase and activation of NFκB through signaling by TNFR1 or TNFR2 or by TNFα and IL-1β has not been previously characterized in detail in adipocytes. The activation of these signaling pathways is of interest because IL-1β and TNFα negatively regulate insulin signaling, and both cytokines suppress genes that enhance glucose uptake in adipocytes [113,122,123,292,296]. Comparing the time frame in which TNFα regulates the expression of genes to the time course of activation of these signaling pathways provides insight regarding the mechanisms responsible for gene regulation. Using this approach, we found striking correlations between the extent to which TNFα was able to maintain prolonged activations of JNK1/2 and p38 SAP kinase and its ability to increase Map4k4 expression (Fig. 2.1 and 2.4). In contrast, the other cytokines we tested exerted only transient effects on these intermediate protein kinases and failed to mimic the stimulatory effects of TNFα on Map4k4 expression (Fig. 2.3). Interestingly, LPS and IL-1β but not IL-6, have also been implicated in decreasing PPARγ levels in brown adipocytes [309]. Considering that these cytokines do not increase Map4k4 expression (Fig. 2.3), our results suggest that TNFα may utilize Map4k4 expression as a unique mechanism to augment its inhibitory actions on PPARγ levels. These data suggest that TNFα is a particularly potent cytokine in regulating adipose function, and supports

previous work indicating it is a powerful negative regulator of adipogenesis and insulin signaling in intact mice and humans [111-115,122,123,293-295].

The signaling mechanism through which TNFα regulates Map4k4 expression is mostly mediated by the TNFR1, while the actions of TNFR2 play little or no role (Fig. 2.7, 2.8 and 2.9). This data is consistent with previous work showing a requirement of TNFR1 for TNFα attenuation of insulin signaling and the enhancement of lipolysis [124,296]. Interestingly, TNFR2 was not required for the impairment of adipogenesis [308]. Furthermore, in that study, expression of a chimeric receptor containing the TNFR2 extra-cellular domain and the TNFR1 intracellular domain, inhibited adipogenesis, suggesting that distinct intracellular signaling pathways are activated by TNFR1 and not by TNFR2 in cultured adipocytes. This concept was confirmed by our findings (Fig. 2.8 and 2.9). Furthermore, in the same body of work, the authors show that the presence of the chimeric receptor suppressed PPARγ levels despite a failure of this receptor to activate NFκB in the presence of TNFα. Thus our findings confirm data from other cell systems in which TNFR2 stimulation failed to produce robust JNK and NFκB activation [303,310,311].

Map4k4 regulation by TNFα may be relevant to functions of this protein kinase beyond its role in adipose biology, especially to its potential regulation of cell motility. For example, Map4k4 is necessary for the epithelial to mesenchymal transition during mouse embryo gastrulation, as evidenced in Map4k4 null mice [271,274]. Although these mice die in the embryonic stage, it is possible to show that the mesoderm is properly specified in these mouse embryos. Instead, the defect resides in the inability of these

mesenchymal precursor cells to migrate [274]. Additionally, TNFα has been proposed to positively regulate the epithelial to mesenchymal transition in a number cell systems [312,313], potentially suggesting a role for Map4k4. Furthermore, Map4k4 was also recently shown to be necessary for the migration of cancer cells [272]. Considering that malignancies like colon cancer require an epithelial to mesenchymal transition, and that this transition is positively regulated by TNFα[312], a potential role for Map4k4 regulating cancer invasion is plausible. These and other recent studies indicating that Map4k4 negatively regulates cellular adhesion and promotes cellular migration[272,274,288] suggests that Map4k4 may in some way modulate cytoskeletal elements or other cellular components necessary for cell motility. Thus, in addition to potentially regulating cancer cell invasion, the regulation of Map4k4 may be important in cells that require motility for proper function, such as cells of myeloid lineages. These cells are known to use a transcription factor network similar to adipocytes to regulate gene expression and even the production of cytokines [314,315]. Thus it is possible that TNFα may increase Map4k4 expression in other cell types to elicit multiple biological responses, including metastasis of cancer cells.

In summary, we have shown here that TNFα is unique among a number of cytokines in its ability to increase the expression of a major element within its own acute signaling pathway, the protein kinase Map4k4. Map4k4 is in turn unique in its response to TNFα, among many Map kinases tested for this response. Furthermore, Map4k4 is distinctive among many Map kinases tested in its ability to modulate insulin sensitivity in cultured adipocytes, as does TNFα [275]. The increase in Map4k4 expression in response to TNFα is mediated through a signaling pathway elicited selectively by TNFR1

activation leading to cJUN and ATF2 activation. Understanding the underlying molecular mechanisms whereby TNFα regulates Map4k4 expression may thus provide insight into new therapies for multiple disease states, including cancer and diabetes.

Limitations and Future Perspectives

Although insights into the mechanisms by which TNFα increases MAP4K4 expression were elucidated in this study, numerous questions still remain. For example, ATF-2 and cJUN were necessary for the full TNFα mediated increase in adipocyte MAP4K4 expression. However, a direct interaction of these transcription factors on the MAP4K4 promoter was never demonstrated. It remains possible that MAP4K4 expression is indirectly controlled by ATF-2 and cJUN. Therefore, experiments involving chromatin immuno-precipitation should be utilized to establish an ATF-2 and cJUN interaction on the MAP4K4 promoter. Potential binding sites should also be tested utilizing a luciferase reporter system. In these experiments sequence specific mutations can be made to verify the exact elements regulated by TNFα.

The role of other transcription factors in controlling MAP4K4 expression, such as NFκB, is also another remaining question. As mentioned earlier, NFκB is a major transcriptional regulator of gene expression in response to TNFα. Thus, testing the role of NFκB in controlling MAP4K4 expression following TNFα treatment is of interest. A potential pitfall with this approach is the requirement of NFκB to induce survival gene expression which counters the effects of TNFα mediated caspase activation[316]. Hence,

to simply inhibit NFκB during TNFα treatment results in rapid cell death. Therefore, the mutation of potential NFκB interacting sites on a MAP4K4 luciferase promoter construct might be a more reasonable approach. Future work will no doubt identify other transcription factors that mediate the TNFα increase in MAP4K4 expression.

Finally, we hypothesized that sustained activation of ATF-2 and cJUN potentiate MAP4K4 expression following TNFα treatment. The data to support this hypothesis is correlative as we measured the phosphorylation of ATF2 and cJUN activation residues following treatment with TNFα, which increases MAP4K4 expression, and IL-1β which does not change MAP4K4 expression. Perhaps this question can better be addressed by identifying the phosphatases that negatively regulate ATF-2 and cJUN activation in response to IL-1β treatment. Silencing their expression may result in sustained activation of ATF-2 and cJUN following IL-1β treatment along with an increase in MAP4K4 expression. Overall, the experiments suggested here could further enhance our knowledge of MAP4K4 regulation by TNFα. In the future, work regarding the role of TNFα on MAP4K4 expression should address these remaining questions.

CHAPTER III: Orally delivered siRNA targeting macrophage MAP4K4 suppresses systemic inflammation

Disclaimer: All experiments were a collaborative effort between Myriam Aouadi and me. Sarah Nicoloro helped with the mouse work and staining and analysis of cells and tissues for microscopy. Mengxi Wang and My Chouinard helped with the mouse work, RNA isolation and real time PCR. All yeast particles were prepared and loaded by Gary Ostroff and Ernesto Soto. Myriam Aouadi and I loaded the siRNA into the GeRPs. Myriam Aouadi and I performed all statistical analysis done in this chapter.

This Chapter is in the format that we submitted to Nature and is under second review at the time of this dissertation:

Myriam Aouadi*, **Gregory J. Tesz***, Sarah M. Nicoloro , Mengxi Wang, My Chouinard, Ernesto Soto, Gary R. Ostroff, Michael P. Czech. 2008. Orally delivered siRNA targeting macrophage MAP4K4 suppresses systemic inflammation.
** These authors contributed equally to this work*

Abstract

Gene silencing by double stranded RNA, denoted RNAi, is mediated by specific targeting and degradation of messenger RNAs, and represents a novel paradigm for rational drug design. However, the potentially transformative therapeutic applications of RNAi have been stymied by a key obstacle—selective, safe delivery to target tissues *in vivo*. Here we report the engineering of encapsulated siRNA that potently silences genes in mouse macrophages *in vitro* and *in vivo*. Using this method we discovered that the mitogen activated protein 4 kinase 4 (MAP4K4) is a novel mediator of

inflammatory responses. Oral delivery of as little as 10 μg/kg siRNA depleted MAP4K4 in macrophages recovered from the peritoneum, spleen, liver and lung in mice. Importantly, silencing MAP4K4 in macrophages protected mice from lipopolysaccharide-induced lethality by inhibiting tumor necrosis factor alpha and interleukin-1 beta production. These results provide a novel strategy for oral delivery of siRNA to attenuate inflammatory responses in human disease.

Introduction

The discovery that short sequences of double stranded RNA can cause depletion of cognitive RNA transcripts in eukaryotic cells has greatly expanded our understanding of gene regulation[242]. The specificity and potency of gene silencing by RNA interference (RNAi) is facilitated by cellular machinery that mediates these actions. For therapeutic applications, double stranded short interfering RNA (siRNA) oligonucleotides are relatively nontoxic, readily designed for high specificity, and need not be restricted to genes that encode proteins that bind small molecule drugs[245,317]. Thus RNAi can be targeted to all genes that encode protein sequences. Additionally, siRNAs are designed to minimize the interferon response associated with exposure of cells to long sequences of double stranded RNA[318,319]. Despite these properties, obstacles to *in vivo* delivery of siRNA are numerous and daunting. These include rapid degradation of siRNA oligonucleotides in extracellular environments, rapid excretion through the kidney, and low permeability through tight junctions and across cell surface membranes[266,267]. Creative efforts have addressed some of these problems, and a few clinical trials are underway[268]. However, a key goal in the field is to develop techniques that orally deliver siRNA-mediated gene silencing to specific target tissues and cell types.

In our studies designed to achieve this goal, we chose the macrophage as a potential target because it controls inflammatory responses associated with such major diseases as rheumatoid arthritis, colitis, and atherosclerosis. As a specialized host defense cell, the macrophage is a validated pharmaceutical target that contributes to pathogenesis through secretion of such inflammatory cytokines as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β)[320,321]. To accomplish oral delivery of siRNA to macrophages in mice, we took advantage of a distinctive characteristic of micron-sized particles of β1,3-D-glucan that allows their passage through M cells in Peyer's patches in the intestinal wall (Supplementary Fig. 3.1) to the underlying gut associated lymphatic tissue (GALT)[322,323]. Following transcytosis of such β1,3-D-glucan particles into the GALT, they undergo phagocytosis by resident macrophages and dendritic cells via the dectin-1 receptor and perhaps other beta glucan receptor-mediated pathways[324,325]. GALT macrophages traffic away from the gut and infiltrate other reticuloendothelial system tissues, such that over time a significant proportion of total body macrophages contain ingested glucan particles[323,326].

Materials and Methods

Preparation of hollow β1,3-D-glucan shells and siRNA encapsulation

The siRNA was incorporated into the interior of hollow glucan shells to make glucan encapsulated siRNA Particles (GeRP) by a layer by layer synthesis strategy. Briefly, empty glucan shells (previously shown to be non-immunogenic)[327] were prepared from *Saccharomyces cerevisiae* and fluorescently labeled. Then RNA was

absorbed into dry hollow glucan shells and encapsulated cationic complexes formed by cationic polymer trapping. Negatively charged fluorescently labeled siRNA was absorbed onto the positively charged complex surfaces inside the glucan shells and finally coated with a cationic polymer layer to produce multi-layered GeRP formulations.

Cell culture and GeRP treatment

10 week-old C57BL6/J male were *i.p.* injected with 1 ml 4% thioglycollate broth. After 1-3 days, PECs were isolated and incubated for 48 h with GeRPs and FL-GeRPs at a 10:1 particle-to-cell ratio, as previously determined[323]. For microscopic analysis, cells were incubated with a F4/80 primary antibody antibody (AbD-Serotec, Raleigh, NC) followed by an Alexafluor633 secondary (Invitrogen Molecular Probes, Eugene, OR). Nuclei were stained with either Hoescht 33342 or DAPI as denoted in the figure legends.

Animals.

GeRP i.p. injection. 10 week-old C57BL6/J male mice were *i.p.* injected daily for 3 days, from day 1 to day 3, with 2 x 10^9 GeRPs/kg (4mg/kg) containing 10 μg/kg Scr or MAP4K4 siRNA. *GeRP gavage.* 10 week-old C57BL6/J male mice were administered 4 x 10^9 GeRPs/kg (8mg/kg) containing 10 μg/kg Scr or MAP4K4 siRNA by daily oral gavage for 8 days.

LPS Lethality Test.

11 week-old C57BL6/J male mice treated with GeRPs containing Scr or MAP4K4 siRNA were *i.p.* injected with a single dose of D-galactosamine (25 mg per mouse)

followed by an *i.p*. injection of *E. coli* LPS (0.25 μg per mouse). LPS and D-GalN were solubilized in sterile PBS. Animals were monitored for lethality for 24 h after LPS/D-GalN challenge. Blood and peritoneal fluid were collected at 1.5 and 4 h after LPS/D-GalN injection for TNF-α level measurements.

All procedures involving animals were approved by the Institutional Animal Care and Use Committee at University of Massachusetts Medical School.

Preparation of glucan shells

Glucan shells were prepared as previously described[328]: *S. cerevisiae* cells (100 g Fleishmans Bakers yeast, AB Mauri™ Food Inc., Chesterfield, MO) were suspended in 1 liter 1 M NaOH and heated to 80 °C for 1 h. The insoluble material containing the yeast cell walls was collected by centrifugation at 2000xg for 10 minutes. This insoluble material was then suspended in 1 liter of water and brought to pH 4-5 with HCl, and incubated at 55 °C for 1 h. The insoluble residue was again collected by centrifugation and washed once with 1 liter of water, four times with 200 ml isopropanol and twice with 200 ml acetone. The resulting slurry was placed in a glass tray and dried at room temperature to produce 12.4 g of a fine, slightly off-white powder. Glucan shells are 2-4 microns in size, hollow, porous microspheres consisting primarily of beta 1,3-D-glucan and typically contain $5x10^{11}$ particles/g[329]. All chemicals, unless otherwise indicated were from VWR (West Chester, PA), Fisher (Waltham, MA) or Sigma (St. Louis, MO) and used without further purification.

Fluorescein labeling of glucan shells

Glucan shells (1 g) were suspended in 100 ml 0.1 M sodium carbonate buffer (pH 9.2), collected by centrifugation at 2000xg for 10 minutes and resuspended in 100 ml 0.1 M carbonate buffer (pH 9.2). 5-(4,6 - Dichlorotriazinyl) aminofluorescein (Molecular Probes, Eugene, OR) at a concentration of 1 mg/ml in DMSO was added to the buffered glucan shell suspension (10% v/v) and the reaction was mixed at room temperature in the dark overnight. Tris buffer (2 ml 1M, pH 8) was added and the reaction mixture was stirred for additional 15 minutes at room temperature to quench free fluorescent labeling reagent. The fluorescently labeled glucan shells were collected by centrifugation at 2000xg for 10 minutes and washed with sterile pyrogen-free water until the color was removed. The glucan shells were then dehydrated by four washes with absolute ethanol, two washes with acetone and dried in the dark at room temperature. The resulting powder was ground to a fine bright yellow powder to produce ~ 1 g of FL-glucan shells.

Preparation of Glucan Encapsulated siRNA Particles (GeRPs)

Dry glucan shells or FL-glucan shells were mixed with a volume of the anionic core polymer yeast RNA (Sigma;10 mg/ml in 50 mM Tris HCl pH 8, 2 mM EDTA and 0.15M NaCl (TEN)) to minimally hydrate the glucan shell, and incubated for 2 h at room temperature to allow the glucan shell to swell and adsorb the yeast RNA solution as previously described[328]. Neutral PEI (Aldrich; 25Kd branched PEI; 2 mg/ml in TEN, pH 7) was added in excess to form glucan shell-encapsulated RNA complexes, and the shells resuspended by homogenization or sonication. PEI adsorption and complex formation was allowed to proceed for at least 1 h at room temperature. The glucan shell-encapsulated cationic complexes were centrifuged and shells resuspended in 70%

ethanol, washed three times in 0.9% saline, resuspended, counted using a hematocytometer at 200X magnification, diluted to $1x10^8$ shells/ml in 0.9% saline, and stored at -20°C. The binding of an siRNA layer to the cationized complex surfaces inside the uncharged glucan or FL-glucan shells was accomplished as previously described for DNA binding[328], by preparing a suspension of glucan shell-encapsulated cationic complexes, ($1x10^8$ particles/ml), siRNA at indicated concentrations, and 0.9% sterile, pyrogen-free saline (total volume = 75 μl). The suspension was incubated for 2 hours at room temperature to allow for siRNA binding to the cationic complex surfaces, and neutral PEI (25 μl 0.01% w/v) in 0.9% saline was added to trap and coat the bound siRNA inside the uncharged glucan shells. The efficiency of siRNA binding to the glucan shell-encapsulated cationic complexes was demonstrated to be >95% of input siRNA by measuring unbound Dy547 labeled fluorescent siRNA in the supernatant (data not shown). The homogeneity of shells formulated using this Layer-by-Layer synthesis method has been previously characterized by FACS analysis using fluorescent complex components[328]. For animal experiments the siRNA formulation process was carried out at 20-fold higher concentration of all components, and following the addition of the final layer of PEI the GeRP formulations were collected by centrifugation at 2000xg for 10 minutes and resuspended in sterile pyrogen-free saline to deliver 200 μg of GeRP formulation in 200 μl. The concentrated GeRP formulations were briefly sonicated to break up any aggregates and stored at -20 °C until use.

Tissue macrophage isolation

10 week-old C57BL6/J male mice were administered 200 µg GeRPs by daily oral gavage for 8 days. On day 9, mice were *i.p.* injected with thioglycollate, and PECs and cells from spleen, liver, lung and muscle isolated on day 10. Spleen, liver, lung and muscle tissues were cut into small pieces, washed with Dulbecco's phosphate-buffered saline (PBS) and digested at 37 ºC for 30 min. with agitation using 5 mg/ml collagenase. Digested tissues were then filtered through a 70 µm pore nylon mesh filter and centrifuged 10 min at 1300 rpm. Cells were plated in plastic dishes for 2-3 hours in DMEM, 10% fetal bovine serum. The cells were then washed with PBS to remove non-adherent cells and the adherent cells were used for Real Time PCR.

For confocal analysis, mice were gavaged with a single dose of 400 µg GeRP in a 200 µl volume and tissue macrophages were isolated 24 hours after, as described above. Cells were incubated with a F4/80 primary antibody (AbD-Serotec, Raleigh, NC) followed by an Alexafluor633 secondary antibody (Invitrogen Molecular Probes, Eugene, OR). Nuclei were stained with DAPI.

Isolation of RNA and Real Time PCR

RNA isolation was performed according to the Trizol Reagent Protocol from (Invitrogen, Carlsbad CA). The cDNA was synthesized from 1 µg of total RNA using iScript cDNA Synthesis Kit according to the manufacturer's instructions from (Bio-Rad, Hercules CA). For real time PCR, synthesized cDNA forward and reverse primers along with the iQ SYBR Green Supermix (Bio-Rad, Hercules CA) were run on the MyIQ Realtime PCR System (Bio-Rad). Sequences of the primers used are listed in the table below and were designed with Primer Bank **(Supplementary Table 4)**. The ribosomal

mRNA, 36B4 was used as an internal loading control, as its expression did not change over a 24 h period with the addition of LPS, TNF-α or the silencing of the genes used in this study.

ELISA assay

Mouse TNF alpha ELISA kit was used to measure concentrations of mouse TNF-α in the PEC supernatant, plasma and peritoneal fluid. Mouse Interferon gamma ELISA kit was used to measure concentrations of mouse Interferon gamma in plasma. ELISA kits were purchased from Pierce (Rockland IL) The ELISA immunoreactivity was quantified by measuring O.D at 450 nm and quantitated by a standard curve.

AST and ALT measurement

Evaluation of liver toxicity was performed by determining the enzymatic activity of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum samples using a commercial kit (Fisher Scientific, Hampton, NH) according to the manufacturer's instructions.

Histology and TUNEL assay

Tissue sections were stained with F4/80-AlexaFluor405 antibody (AbD-Serotec, Raleigh, NC) and hematoxylin stained. TUNEL assay was performed on liver sections from mice challenged with LPS/D-GalN according to the manufacturer’s instructions (Upstate, Lake Placid NY). TUNEL images were obtained using a Zeiss Axiovert 200 inverted microscope equipped with a Zeiss AxioCam HR CCD camera with 1,300 × 1,030 pixels basic resolution and a Zeiss Plan NeoFluar 20×/0.50 Ph2 (DIC II) objective.

Tissue and cell images were obtained with a Solamere CSU10 Spinning Disk confocal system mounted on a Nikon TE2000-E2 inverted microscope. Images were taken with a multi-immersion 20x objective with a N.A. 0.75; Oil: W.D.=0.35mm, or a 100x Plan Apo VC objective NA=1.4, Oil: W.D.=0.13mm.

Statistics

The distributional characteristics of the outcomes were evaluated by both a visual inspection of histograms and the Kolmogorov-Smirnoz test performed on model residuals. Transformations by natural logarithms were used in some cases to better approximate a normal distribution and to stabilize variances. The observed effects were evaluated by either one-way or multifactorial analysis of variance (ANOVA). In the presence of significant main and/or interaction effects pairwise comparisons were made using the Tukey (HSD) multiple comparisons procedure with the exception of Figure 3.5F where the Kaplan-Meier analysis was used. Statistical significance of $p < .01$ was determined using Log Rank (Mantel-Cox), Breslow and Tarone-Ware tests. Computations were performed using SAS or SPSS Statistical software packages. The data are presented as means +/- SEM.

Results

We prepared hollow, porous micron-sized shells composed primarily of β1,3-D-glucan by treating baker's yeast with a series of alkaline, acid and solvent extractions to remove cytoplasmic components, as well as other cell wall polysaccharides[328] (Fig. 3.1). Such empty glucan shells are about 2-4 microns in diameter, and can be fluorescently labeled for tracking. Layer by layer synthesis methods were then developed to load

them with siRNA, yielding β1,3–D-Glucan-Encapsulated siRNA Particles (GeRPs), as depicted in Figure 3.1. First, a core complex of anionic material (RNA, DNA or other negatively-charged polymer) is synthesized within the glucan shells by electrostatic complex formation with a cationic polymer such as polyethylenimine (PEI). The positively-charged cores inside the uncharged glucan shells electrostatically absorb the anionic siRNA payload to form GeRPs (Fig. 3.1). Next is added another layer of the cationic polymer PEI, chosen for its effective action as a transfection agent and its relative low toxicity *in vivo*[330]. Additional layers of anionic siRNA and cationic PEI can be applied to synthesize multi-layered GeRPs composed of single or multiple siRNAs (not shown). The anionic siRNA within GeRPs is bound between cationic polyethylenimine (PEI) layers through electrostatic interactions in a pH-dependent manner. Upon phagocytosis by macrophages, GeRPs traffic to the endosomal compartment, where the acidic pH changes the layers' charge. This promotes siRNA release from the multi-layered core through the porous GeRP wall and endosomal membrane into the macrophage cytoplasm.

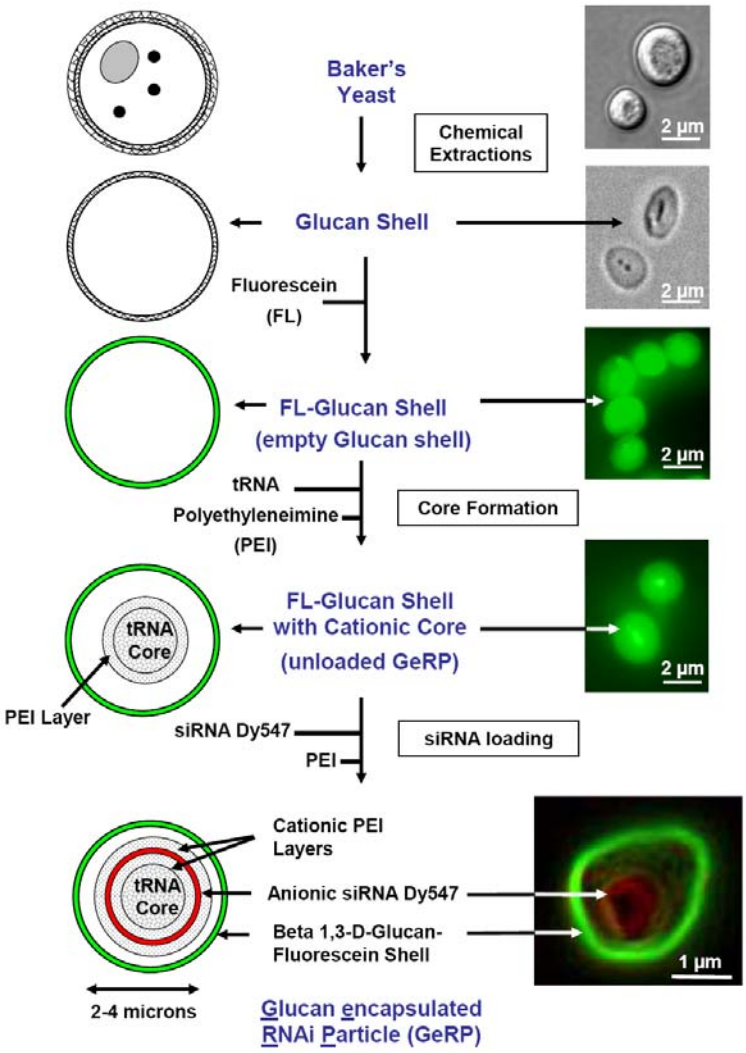


Figure 3.1. Production of fluorescent Glucan encapsulated RNAi Particles (GeRP). Glucan particles were purified from Baker's yeast by a series of alkaline and solvent extractions hydrolyzing outer cell wall and intracellular components yielding purified, porous 2-4 micron, hollow beta 1,3-D-glucan particles (diagram of particles, left; procedure, middle; microscopy of particles, right). Empty glucan particles were then labeled with fluorescein as described in the supplementary methods to track the glucan shells. Cationic cores were synthesized inside the glucan shells by absorbing a sub-saturating volume of yeast tRNA to partially swell the particles followed by reaction with an excess of polyethyleneimine (PEI) to form encapsulated complexed cationic cores as evidenced by the phase distinct structures within the fluorescent glucan shells. Layer by layer synthesis methods were then used to absorb anionic Dy547-labeled siRNA onto the cationic surface of the cores followed by a final coat of PEI as seen by the fluorescent siRNA (red) inside the fluorescent glucan shells (green) in the confocal image on the far right bottom. All images were produced by Ernesto Soto and Gary Ostroff.

To test GeRP formulations for siRNA-mediated gene silencing in macrophages, mouse peritoneal exudate cells (PECs) were prepared after intraperitoneal (*i.p.*) thioglycollate injection, as described in *Methods*. Preliminary experiments using a variety of siRNAs encapsulated within GeRPs showed extensive phagocytosis of GeRPs by the primary macrophages and significant gene silencing. For example, two siRNA oligonucleotides against TNF-α were highly effective in markedly inhibiting its expression in macrophages stimulated with lipopolysaccharide (LPS) (Supplementary Fig 3.2, bottom panel). We then used this system of gene silencing in primary macrophages to test whether candidate intracellular signaling proteins might control TNF-α expression. One of these was the Mitogen Activated Protein 4 Kinase 4 (MAP4K4), a germinal center protein kinase that we and others found facilitates TNF-α signaling itself[183,275,276,331]. In these experiments, glucan shells were first derivatized with a green fluorescein (FL) probe. They were then loaded with Scrambled (Scr) or MAP4K4 siRNA (oligo 1 in Supplementary Table 1) coupled to the red fluorescent probe, Dy547, using the layer by layer synthesis methods to prepare GeRPs as described in *Methods*. PECs were incubated *in vitro* with these dual-labeled Scr siRNA- or MAP4K4 siRNA-containing GeRPs (10:1 particle-to-cell ratio) for 12 hours, and then stained with the macrophage specific F4/80-AlexaFluor350 antibody and the nuclei were stained with Hoescht (Fig. 3.2A; F4/80-AF350/Hoescht; blue stain). Using confocal microscopy at low magnification, about 90% of the macrophages had internalized at least one FL-GeRP, as visualized by fluorescein or Dy547-siRNA fluorescence (Fig. 3.2A, left panels), while most cells had internalized multiple FL-GeRPs (Fig. 3.2A, right panels show 2 cells at 100x magnification). Using the GeRPs

containing MAP4K4 siRNA, a 70-80% knockdown of MAP4K4 mRNA was achieved in 10^6 PECs with as little as 40 pmoles siRNA (Fig. 3.2B), while PBS, unloaded GeRPs or those containing Scr siRNA had no effect (Fig. 3.2C).

In testing the effect of MAP4K4 silencing on the macrophage inflammatory response *in vitro*, we stimulated macrophages with LPS, a major structural component of the outer membrane of Gram-negative bacteria. LPS activates monocytes and macrophages to produce cytokines such as TNF-α and IL-1β that act as endogenous inflammatory mediators[332]. PECs (10^6) were incubated with 10^7 GeRPs containing 40 pmoles of Scr or MAP4K4 siRNA for 48h, and then treated with saline or LPS for an additional 6h before total mRNA was harvested and analyzed by RT-PCR. TNF-α mRNA levels were decreased by 40% in control cells treated with MAP4K4 siRNA-containing GeRPs compared to GeRPs containing Scr siRNA (Fig. 3.2D). Importantly, this MAP4K4 silencing inhibited the LPS-induced TNF-α expression by nearly 50% (Fig. 3.2D), while use of a second siRNA sequence against MAP4K4 in this protocol led to even a more robust knockdown (Supplementary Fig 3.2B). MAP4K4 silencing in PECs also resulted in an average 30% decrease of LPS-induced TNF-α protein secreted into the medium (Fig. 3.2E). GeRPs with Scr siRNA, unloaded GeRPs (GeRPs containing tRNA/PEI cores, but not siRNA) or PBS failed to affect TNF-α expression (Fig. 3.2C) or secretion (Supplementary Fig 3.3), nor did these control treatments have an effect on expression of interferon response genes (Supplementary Fig. 3.10).

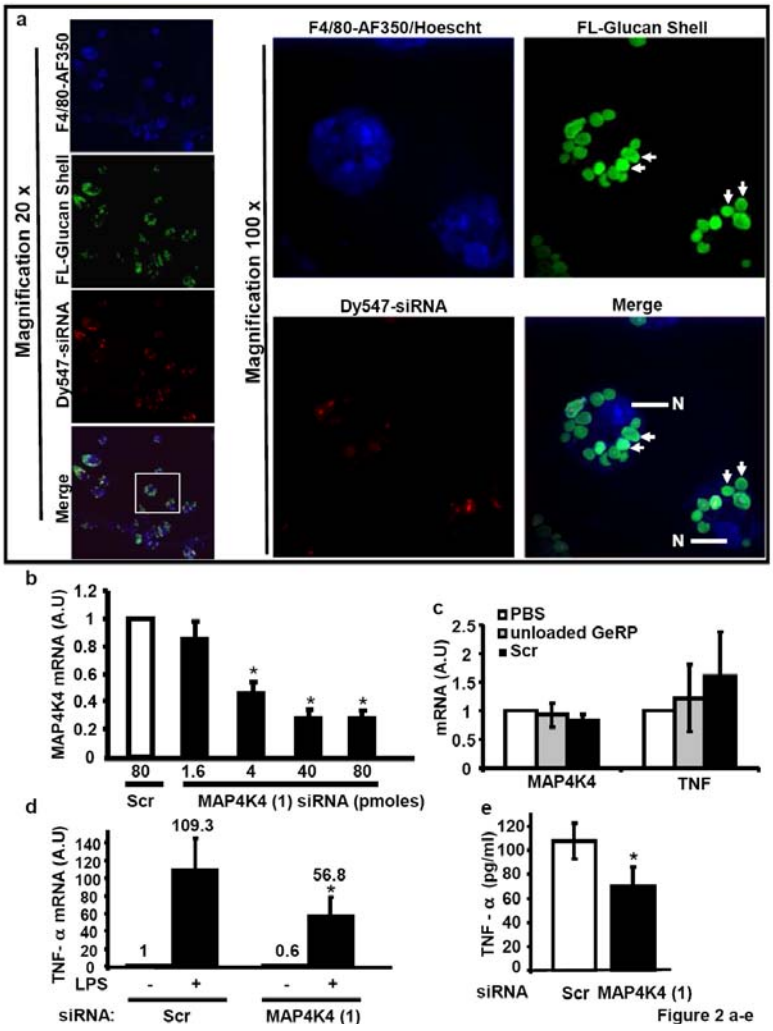


Figure 3.2. GeRPs containing MAP4K4 siRNA silence MAP4K4 expression and inhibit LPS induced TNF-α production in macrophages. (a) Confocal imaging of thioglycollate-elicited PECs treated *in vitro* with FL-conjugated GeRPs (green) loaded with Dy547-labeled Scr or MAP4K4 siRNA (red). Fixed cells stained with F4/80-Alexa-Fluor350 antibody (blue) confirmed that macrophages phagocytosed GeRPs. Nuclei (N) were stained with Hoescht (blue) (Left panels, magnification: 20x and right panel magnification: 100x, arrows denote representative GeRPs). **(b)** MAP4K4 mRNA expression in PECs treated with GeRPs loaded Scr or MAP4K4 (oligo 1) siRNA. **(c)** Expression of MAP4K4 and TNF-α in PECs treated with PBS, unloaded GeRPs (GeRPs with all constituents present except siRNA) or GeRPs loaded with 40 pmoles of Scr siRNA. (**d**) TNF-α mRNA expression and (**e**) TNF-α secretion in PECs treated with GeRPs loaded with 40 pmoles of Scr or MAP4K4 siRNA. Results are expressed in arbitrary units and are the mean ± SEM (n=4). Statistical significance was determined by ANOVA and Tukey post test except for (**e)** where a two tailed student's T-test was performed. *(p <0.01). PECs were prepared by Myriam Aouadi and me. Images were produced by Sarah Nicoloro. Myriam Aouadi and I harvested and analyzed the RNA by real time PCR. ELISA for TNFα and GeRP preparation was performed by Myriam Aouadi.

cJUN-n terminal kinases 1 and 2 (JNK1/2), extracellular signal-related kinase 1/2 (ERK1/2), p38 MAPK and NFκB pathways regulate TNF-α production in various cell types[333,334]. Interestingly, we found that MAP4K4 defines a proinflammatory pathway that activates TNF-α expression independently of the JNK1/2, p38, ERK1/2 and NFκB pathways (Fig. 3.3). Silencing MAP4K4 had no effect on the expression or phosphorylation of JNK1/2, p38 and ERK1/2, ATF2, or cJun. Similarly, no effect of MAP4K4 depletion on the degradation of the NFκB pathway regulator, IκBα in response to LPS was observed. Thus, MAP4K4 knockdown in primary macrophages failed to affect LPS-induced phosphorylation of these protein kinases, or LPS-induced degradation of IκBα in spite of its inhibition of TNF-α expression (Fig. 3.3). These data demonstrate that MAP4K4 is a novel target for suppression of TNF-α expression in LPS-induced macrophage inflammatory responses, in addition to its previously known role in mediating some effects of TNF-α.

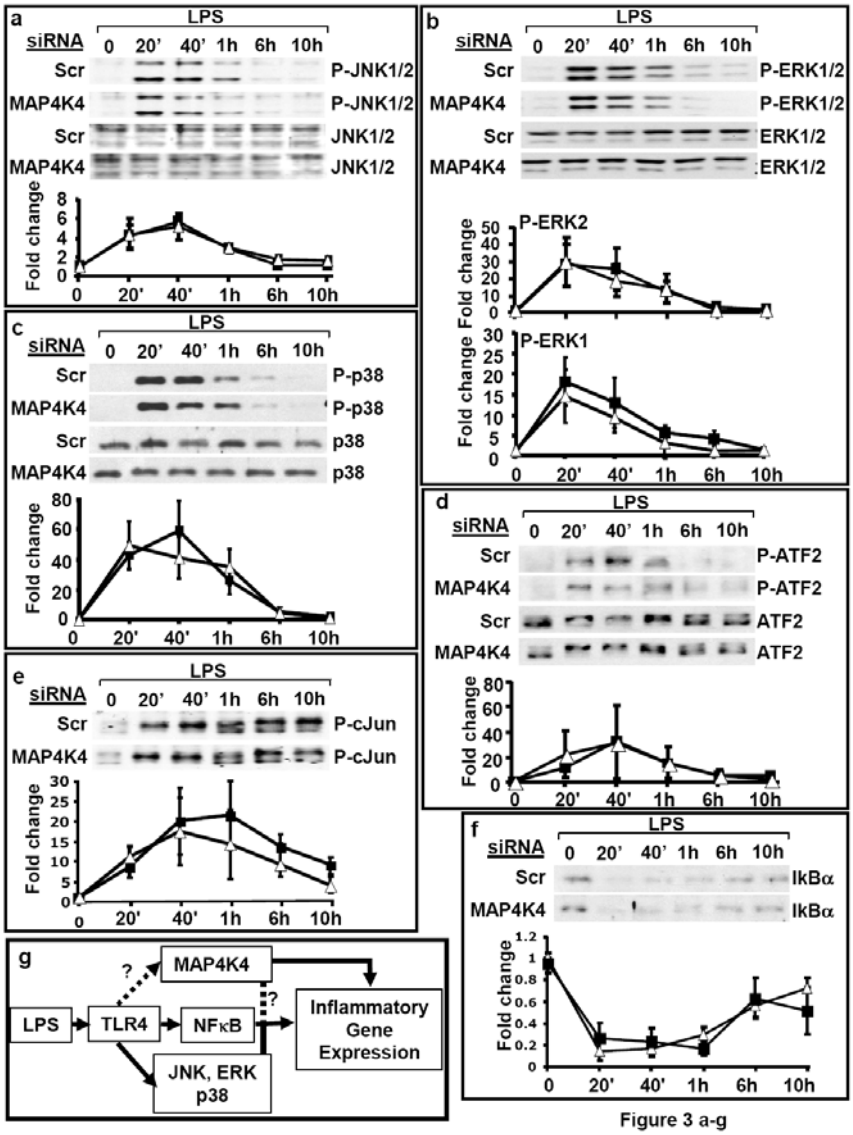


Figure 3 a-g

Figure 3.3. **MAP4K4 silencing attenuates TNF-α expression but not LPS activation of MAP kinase and NFκB signaling pathways**. PECs were treated with GeRPs loaded with 40 pmoles of Scr or MAP4K4 (oligo 1) siRNA, and 48 hours later cells were treated with 1 µg/ml LPS for the indicated amounts of time. Cell lysates were analyzed by Western blot for phospho- and total **(a)** JNK1/2, **(b)** ERK1/2, **(c)** p38MAPK, **(d)** ATF-2; and **(e)** phospho-cJUN and **(f)** total IκBα. Representative blots are shown from 3 different experiments. Graphs show the mean of densitometry analysis ± SEM of the immunoblot signals and are expressed in arbitrary units (n=3) Black squares (■), Scr-siRNA and white triangle (△), MAP4K4-siRNA. Statistical significance was determined by ANOVA and Tukey post test. Under these conditions, MAP4K4 depletion markedly attenuated TNF-α expression (not shown). (**g**) Schematic diagram of potential MAP4K4 signaling to modulate the expression of inflammatory genes such as TNF-α and IL-1β, indicating it acts independently or downstream of MAP kinases and NFκB. PECs were prepared by Myriam Aouadi and me. I did all western blot data and densitometry.

In order to evaluate GeRPs for oral delivery of siRNA-mediated gene silencing *in vivo*, mice were given Dy547-conjugated Scr or MAP4K4 siRNA (10 μg/kg) contained within FL-GeRPs ($4x10^9$ FL-GeRPs/kg) by oral gavage once daily for 8 consecutive days. The mice were then *i.p.* injected with thioglycollate on day 9 and PECs were isolated on day 10 (see protocol in Fig. 3.4B). Staining the PECs recovered from these mice with the macrophage-specific antibody F4/80-AF633 (Magenta) followed by fluorescence microscopy revealed that the FL-GeRPs containing Dy547-siRNA were indeed efficiently taken up by macrophages (Fig. 3.4A). Co-localization of AlexaFluor633, FL, and Dy547 fluorescent signals in adherent macrophages was readily observed. Strikingly, MAP4K4 mRNA expression as assessed by RT-PCR revealed a 70% knockdown in PECs isolated from mice orally gavaged with MAP4K4 siRNA-GeRPs compared to PECs from control mice treated with Scr siRNA-GeRPs (Fig. 3.4C). This level of knockdown is greater than the relatively lower number of macrophages that could be observed to contain GeRPs in Figure 3.4A, indicating possible degradation of the GeRPs and loss of detectable signal even though siRNA-mediated knockdown persists. This lower number of macrophages containing detectable fluorescent GeRPs compared to the extent of gene silencing could be due to a dilution of the fluorescent tags during breakdown of the glucan shells and dispersal of the siRNA. This issue is under further investigation in our laboratories.

MAP4K4 silencing was also analyzed in macrophages that had migrated to other tissues by dissociating cells from spleen, liver, lung, and skeletal muscle tissues with

collagenase on day 10 of the protocol (Fig. 3.4A), and then isolating an enriched macrophage population (see *Methods*). Significant depletions of about 50%, 80% and 40% in MAP4K4 mRNA levels were observed in these macrophage-enriched cells isolated from spleen, liver and lung tissues, respectively, in mice treated with MAP4K4 siRNA-GeRPs compared to the control mice treated with Scr siRNA-GeRPs (Fig. 3.4C). Interestingly, no silencing of MAP4K4 expression was observed in macrophage-enriched cells derived from skeletal muscle (Fig. 3.4C). In a parallel experiment, we could identify GeRP-containing macrophages isolated from spleen, liver and lung tissues of mice orally gavaged with fluorescently labeled GeRPs (Fig. 3.4A), bottom panel). Again, only a small proportion of macrophages enriched from these tissues contained GeRPs when examined by confocal microscopy (not shown). In other experiments, tissue sections were also prepared and analyzed by fluorescence microscopy for the presence of macrophages containing FL-glucan shells. These studies revealed infiltration of spleen, liver and lung with macrophages containing FL-glucan shells (Supplementary Fig. 3.4B)**,** which could be seen at higher magnification to harbor FL-glucan shells. These cells were identified as macrophages using F4/80 antibody (Supplementary Fig. 3.4B, lower set of panels). However, consistent with the lack of gene silencing in skeletal muscle (Fig 3.4C), GeRP-containing macrophages were not present in this tissue (Supplementary Fig. 3.4B). Taken together, these data indicate that macrophages in the GALT internalize orally absorbed GeRPs, undergo siRNA-mediated gene silencing and move out of the gut to infiltrate tissues throughout the body.

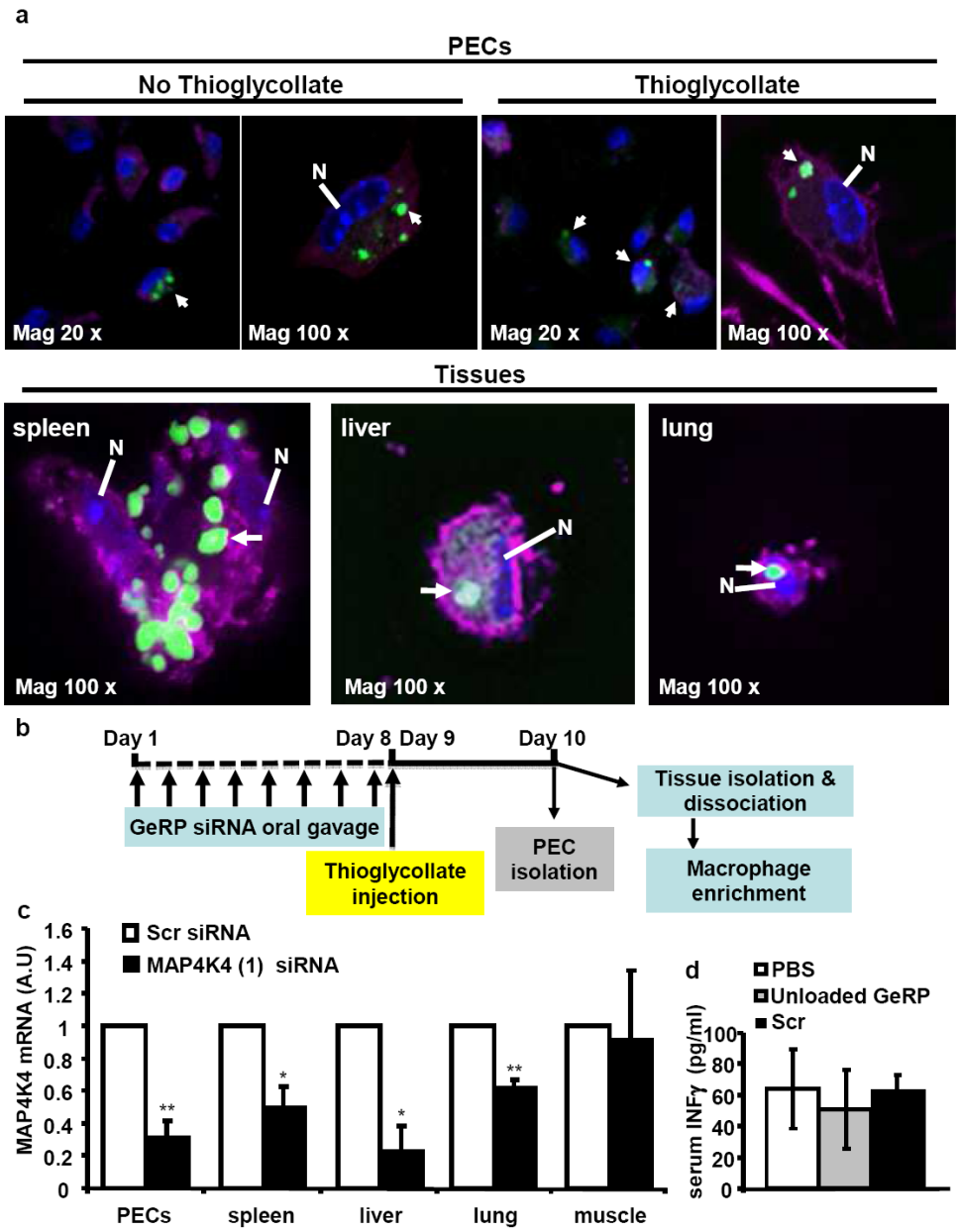


Figure 3.4. Orally administered GeRPs containing MAP4K4 siRNA attenuate MAP4K4 mRNA expression in gut macrophages that traffic into spleen, lung and liver. (a) Confocal microscopy of PECs and macrophage-enriched cells isolated from spleen, liver and lung. For PECs, mice were gavaged with 200 µg GeRPs (FL-labeled shells; Dy547 labeled siRNA) daily for 8 days, injected or not with thioglycollate on day 9 and PECs were isolated on day 10. For tissue macrophages, mice were gavaged with a single dose of 400 µg dual labeled GeRPs and tissues were isolated 24 hours later, followed by preparation of macrophage-enriched cells. Staining with F4/80-AF633 (Magenta) confirmed that some PECs and macrophages from spleen, liver and lung contained GeRPs (green). Nuclei (N) were stained with DAPI (blue). Arrows point to cells containing GeRPs (20x images) or representative GeRPs (100x images). **(b)** Timeline of oral siRNA-GeRP (Scr or MAP4K4, oligo 1) administration, and PEC/tissue

isolation **(c)** Analysis of MAP4K4 expression in PECs and adherent cells from tissues. Results are the mean ± SEM (n=5). **(d)** Mice were gavaged with PBS, unloaded GeRPs or GeRPs loaded with 10 ug/kg of Scr siRNA. Serum INF γ levels were measured by ELISA (n=5). Statistical significance was determined by ANOVA and Tukey post test. Statistical significance was determined by a two tailed student's T-test. ** $p < 0.01$ and * $p < 0.05$. GeRP preparation along with mouse handling, gavages and thioglycollate injections were done by Myriam Aouadi and me. Myriam Aouadi, Sarah Nicoloro, Meng-Xi Wang and I sacrificed and dissected all mice. The images were prepared by Sarah Nicoloro. RNA and real time PCR was done by Myriam Aouadi and me. I did the ELISA for IFNγ.

In order to confirm that gene silencing by orally delivered GeRPs can be mediated by multiple siRNAs, we also gavaged mice with GeRPs containing another MAP4K4 and two TNF-α siRNA oligonucleotides found to be effective on macrophages *in vitro* (Supplementary Fig 3.2). As shown in Supplementary Fig. 3.5, oral gavage of GeRPs containing these three other siRNA oligonucleotides was highly effective in silencing the cognate genes in PECs as well as macrophage-enriched cells isolated from spleen, liver and lung. Importantly, oral gavage of GeRPs containing either siRNA or no siRNA (unloaded GeRPs) did not change interferon gamma levels in serum (Fig 3.4D), consistent with lack of induction of interferon response genes in macrophages treated with GeRPs *in vitro* (Supplementary Fig 3.10). Serum levels of liver enzymes were also all within normal ranges (AST < 255 IU/L; ALT < 77 IU/L)[335] with little effect of any of the treatments (Supplementary Fig. 3.11). Initial experiments indicate that the gene silencing with unmodified siRNA lasted about 8 days following the termination of oral administration of GeRPs under the conditions of these experiments (not shown). Thus, we have demonstrated efficient knockdown of two genes with 4 different siRNA sequences using orally delivered GeRPs (Fig 3.4 and Supplementary Fig 3.2, 3.5).

We also tested the efficacy of MAP4K4 siRNA-GeRPs to silence MAP4K4 expression in macrophages following delivery by *i.p.* injection. Previous studies indicated that 3 daily *i.p.* injections of $2x10^9$ empty glucan shells/kg achieved substantial uptake into macrophages in mice[322,323]. Mice were treated once daily for 3 days by *i.p.* injections of 10 μg/kg Dy547-labeled MAP4K4 siRNA or Scr siRNA in $2x10^9$ FL-GeRPs/kg, and then treated with thioglycollate on day 4 (Supplementary Fig. 3.6A). Fluorescence microscopy revealed PECs contained GeRPs (Supplementary Fig. 3.6B), resulting in a 30% knockdown of MAP4K4 mRNA (Supplementary Fig. 3.6C). These results show that similar to oral delivery, *i.p.* administration of GeRPs causes efficient gene silencing in macrophages *in vivo*.

Since MAP4K4 controls TNF-α expression and secretion by macrophages *in vitro* (Fig. 3.2), we next designed a protocol to test whether oral delivery of MAP4K4 siRNA-GeRPs attenuates cytokine production and LPS-induced lethality in mice (Fig. 3.5A). Preliminary experiments revealed there was no effect of 3 or 10 days of treatment with empty glucan shells by either oral gavage or *i.p.* administration on serum TNF-α levels (Fig. 3.5B). Similarly, we found no effect of oral gavage of GeRPs containing Scr siRNA or no siRNA (unloaded GeRPs containing tRNA/PEI) on serum TNF-α levels prior to LPS treatment compared to PBS administration (Supplementary Figs.3.8A and 3.9A). Remarkably, an 80% decrease in TNF-α expression in PECs was observed in mice orally gavaged with GeRPs containing MAP4K4 siRNA versus Scr siRNA (Fig. 3.5C). This was accompanied by an equally dramatic 80% knockdown of the inflammatory cytokine IL-1β, but not IL-10, or the chemokine receptor CCR2, known to be down-regulated by LPS[336,337]. Importantly, TNF-α siRNA does not silence IL-1β, showing

specificity of this response to MAP4K4 knockdown (Supplementary Fig. 3.7). These results indicate that silencing of MAP4K4 through oral delivery of GeRPs containing MAP4K4 siRNA potently downregulates the expression of the inflammatory cytokines TNF-α and IL-1β in macrophages *in vivo*.

We next measured circulating TNF-α protein levels after challenging mice with injections of LPS and D-galactosamine (D-GalN), known to elicit a powerful inflammatory response. Consistent with previous reports[338], we found that circulating TNF-α levels are strongly elevated 1.5 h after LPS/D-GalN injection and then decrease to basal levels after 4 h. As shown in Figure 3.5D, oral delivery of GeRPs containing MAP4K4 siRNA blocked the elevation in serum TNF-α protein induced by LPS/D-GalN injection. In these mice a significant decrease of TNF-α levels in peritoneal fluid 1.5 h after D-GalN/LPS injection was also observed (Fig. 3.5E). Similarly, serum TNF-α levels in response to LPS were decreased in mice orally gavaged with GeRPs containing other MAP4K4 or TNF-α siRNA oligonucleotides (Supplementary Fig 3.8A), but not with Scr siRNA or unloaded GeRPs compared to PBS treatment (Supplementary Fig. 3.9A). These data demonstrate a striking downregulation of the TNF-α response to an inflammatory stimulus by depletion of MAP4K4 mRNA through oral delivery of GeRPs using two different siRNAs.

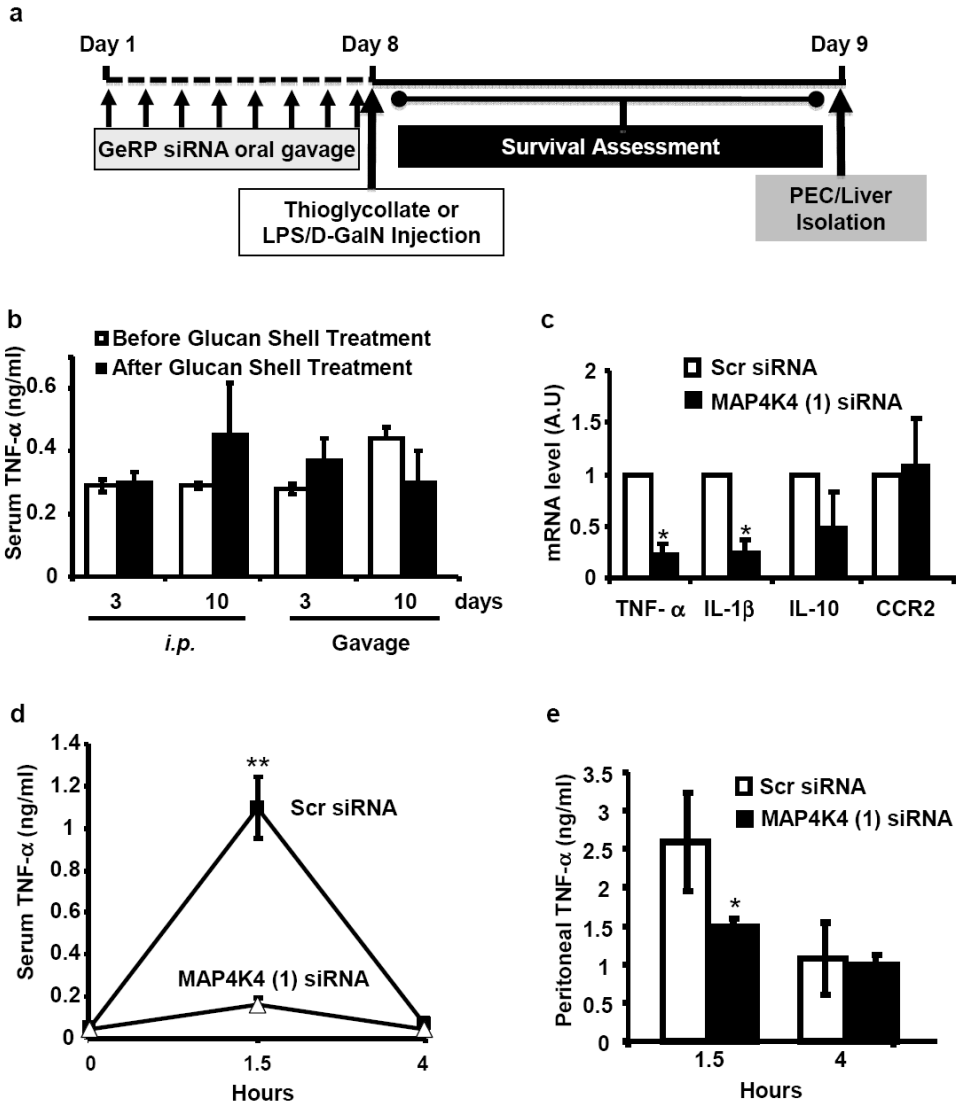


Figure 3.5. MAP4K4 silencing by oral gavage with GeRPs inhibits LPS-induced TNF-α and IL-1β production *in vivo*. (a) Timeline of siRNA and D-galactosamine (D-GalN) /LPS administration. Mice were gavaged once daily with Scr or MAP4K4 siRNA in GeRPs (10 µg siRNA/kg) for 8 days. Four hours after the final gavage, mice were *i.p.* injected with D-GalN, followed by an *i.p.* injection of LPS. **(b)** Basal serum TNF-α levels before and after the administration of empty glucan shells. **(c)** Basal expression of TNF-α**,** IL-1β, IL-10 and CCR2 in PECs isolated from mice orally treated with GeRP siRNA formulations (10 µg/kg). Results are the mean ± SEM (n=3). Statistical significance was determined by a two tailed student's T-test. **(d)** Serum and **(e)** Peritoneal fluid TNF-α levels in siRNA treated mice 1.5 and 4 hours after LPS/D-GalN injection. Results are the mean ± SEM (n=10). Statistical significance was determined by ANOVA and Tukey post test; ** $p < 0.001$ and * $p < 0.05$. GeRP preparations and all animal injections, bleedings and handling were done by Myriam Aouadi and me. Myriam Aouadi, Sarah Nicoloro, My Chouinard and I dissected all mice. RNA and real time PCR was done by Myriam Aouadi and me. Elisa for TNFα was done by Myriam Aouadi.

Lethality observed in LPS/D-GalN-challenged animals is attributed to inflammatory cytokine toxicity and can be mimicked by administration of TNF-α and IL-1β, which synergize with each other[339]. Furthermore, mice lacking the TNF-α receptor TNFRp55 and mice (C3H/HeJ) deficient in TNF-α and IL-1β release are resistant to LPS-induced lethality [204,340]. Normal mice can also be protected from lethal endotoxemia by agents that selectively inhibit TNF-α and/or IL-1β action or release[341,342]. We therefore tested whether such protection is elicited by oral delivery of MAP4K4 siRNA-GeRPs using the protocol in Figure 3.5A. After daily oral gavage for 8 days with Scr siRNA or MAP4K4 siRNA GeRPs (10 μg siRNA/kg), mice were injected *i.p.* with 25 mg of D-GalN followed by 0.25 μg of LPS. As shown in Figure 3.6A, 90% of the control mice treated with Scr siRNA-containing GeRPs died between 4 and 8 h. In contrast, 50% of the mice treated with MAP4K4 siRNA containing GeRPs survived for 8 h after LPS/D-GalN injection. Moreover, 40% of the mice treated with MAP4K4 siRNA containing GeRPs survived the LPS challenge (Supplementary Table 3.2). This difference between administration of Scr versus MAP4K4 siRNA was highly statistically significant using Kaplan-Meier analysis and three independent statistical tests ($p<0.01$) (Supplementary Table 3.3). Similar data were obtained with the alternate MAP4K4 siRNA (oligo 2) and two TNF-α siRNA species (Supplementary Fig. 3.8B), although effects were smaller with the latter.

In murine models, it is well accepted that the lethal effects of LPS/D-Ga1N challenge are due to hepatocyte apoptosis[343]. Thus, we employed TUNEL assays to detect apoptosis in liver of mice treated with Scr or MAP4K4 siRNA-GeRPs, 4 and 28 h

post D-GalN/LPS injection. Hepatocyte apoptosis in response to LPS injection was strongly attenuated by MAP4K4 siRNA-GeRPs (Fig. 3.6B). Furthermore, serum insulin and glucose levels were not significantly different among treatments (Supplementary Fig. 12). Thus, silencing of MAP4K4 expression by oral gavage of MAP4K4 siRNA-GeRPs significantly protects mice from D-GalN/LPS-induced lethality through inhibition of TNF-α and IL-1β production in macrophages.

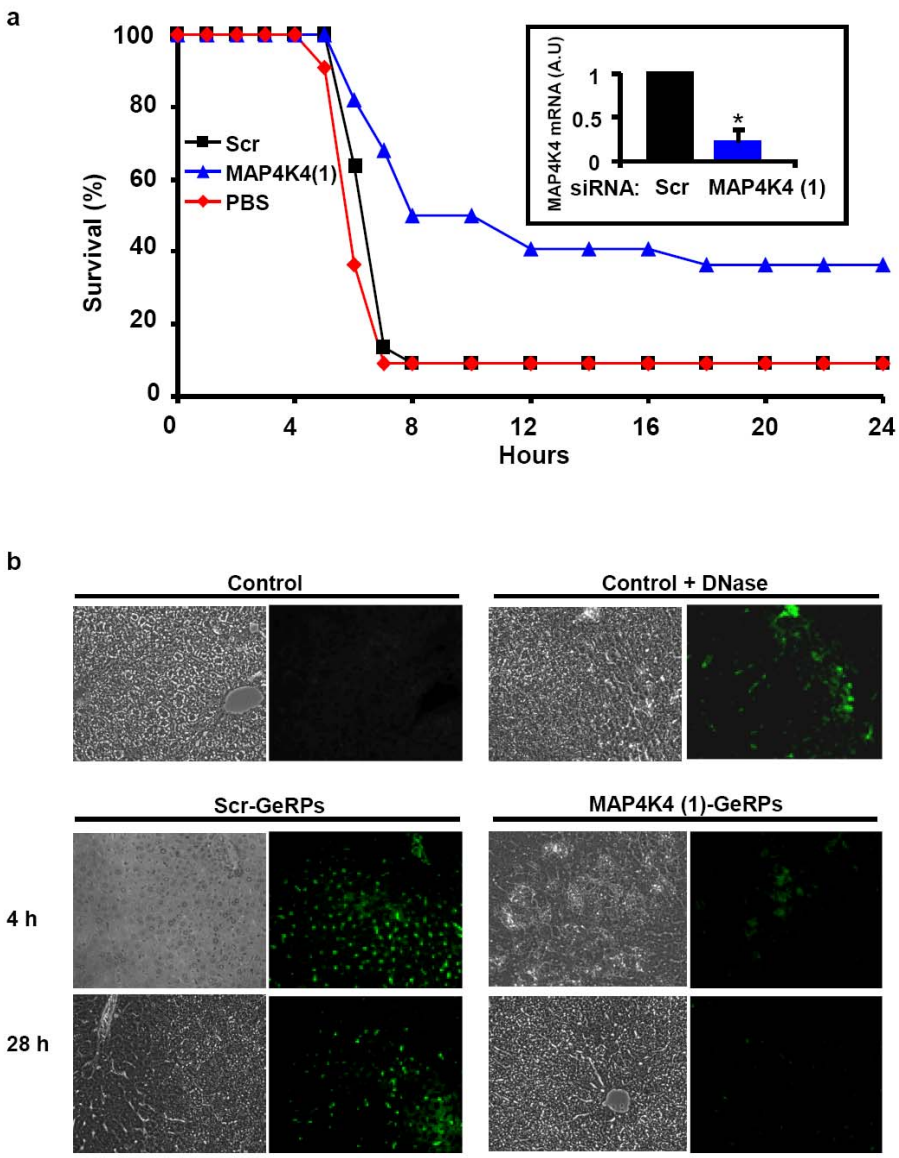
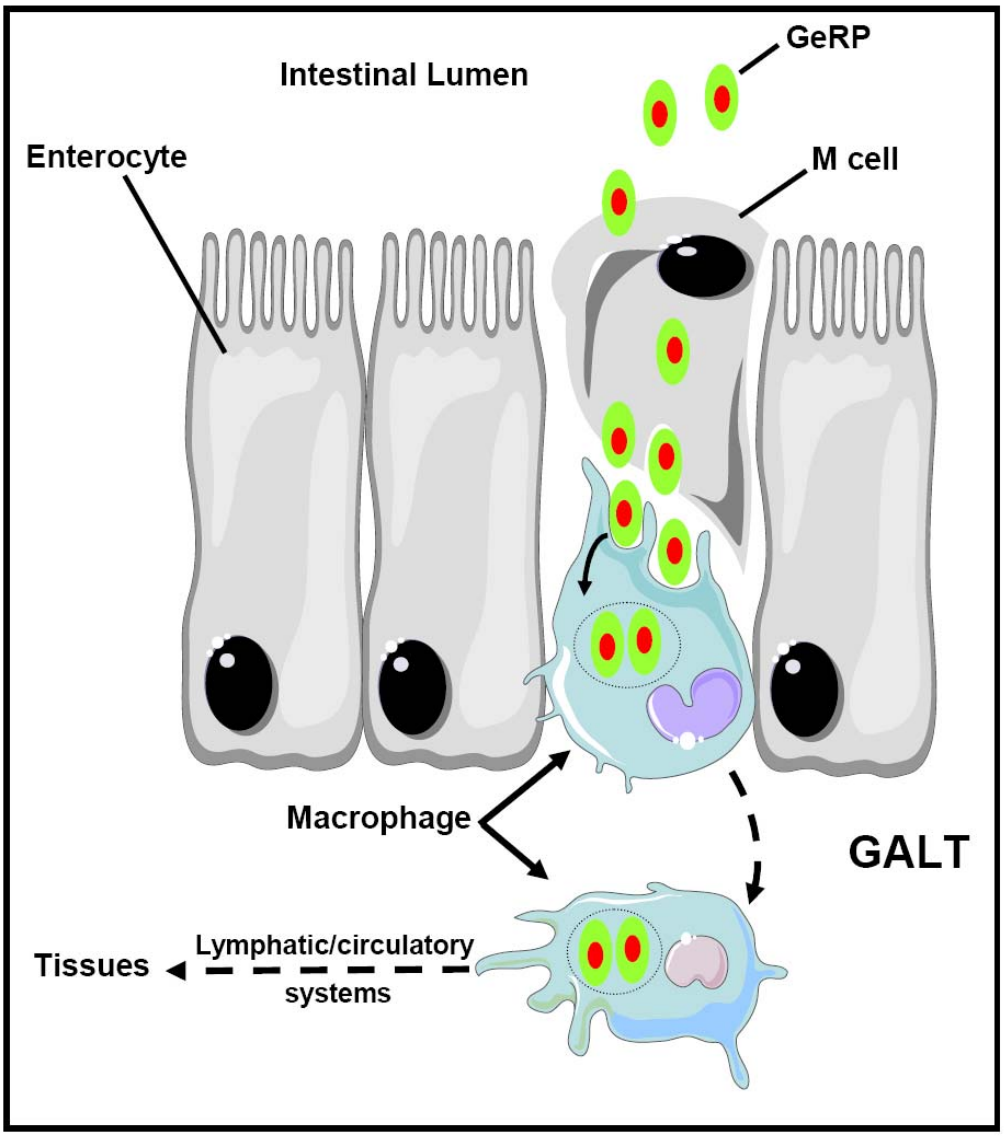
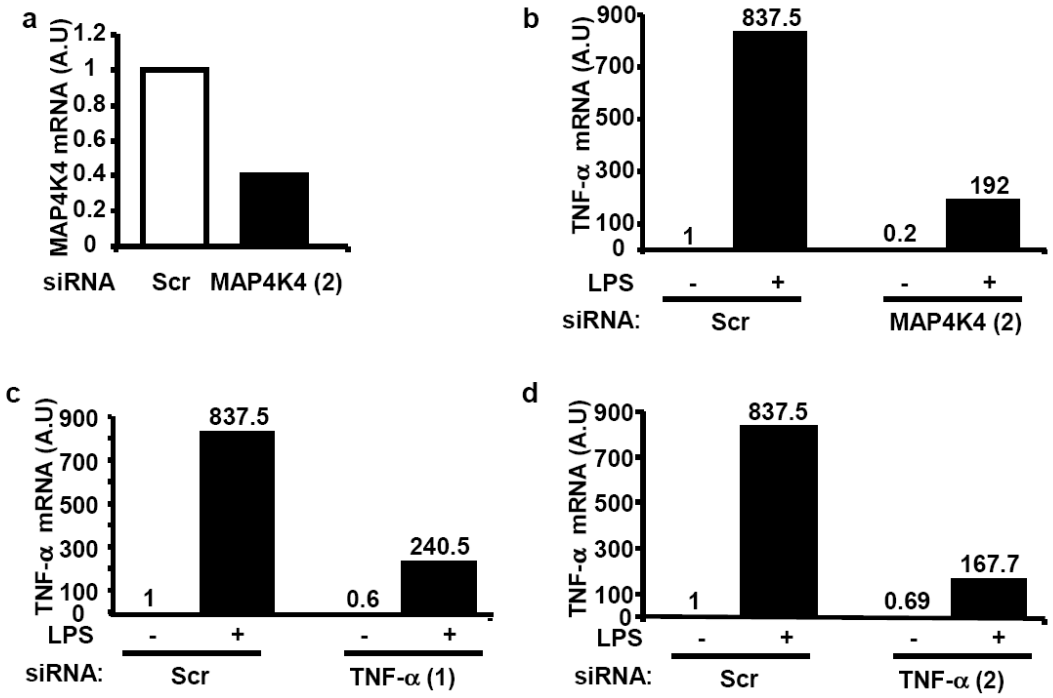


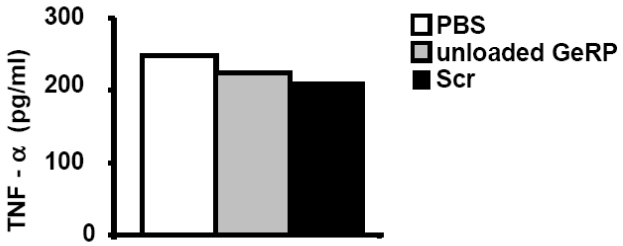
Figure 3.6. MAP4K4 silencing by orally delivered GeRPs inhibits mouse LPS-induced lethality. (**a**) Percent survival of mice orally treated with PBS or GeRPs loaded with Scr or MAP4K4 siRNA, followed by LPS/D-GalN injection. Survival was assessed every hour for 24 hours. Statistical significance was determined using Kaplan-Meier analysis and Mantel-Cox testing ($p<0.01$)(see also Supplementary Table 3.3). Additional groups of three mice treated orally with siRNA GeRPs but without LPS were used in each experiment to assess the MAP4K4 knockdown in the PECs (inset). Results are the mean of three independent experiments (PBS, n=11 mice; Scr and MAP4K4, n=22 mice). (**b**) Silencing MAP4K4 inhibits LPS/D-GalN -induced apoptosis in liver 4 and 28 hours after LPS/D-GalN injection. Livers from untreated mice were treated with or without DNase and used as positive and negative controls, respectively. GeRP preparations and all animal injections, observations and handling were done by Myriam Aouadi and me. Myriam Aouadi, Sarah Nicoloro, and I dissected all mice. Histological images were done by Sarah Nicoloro.



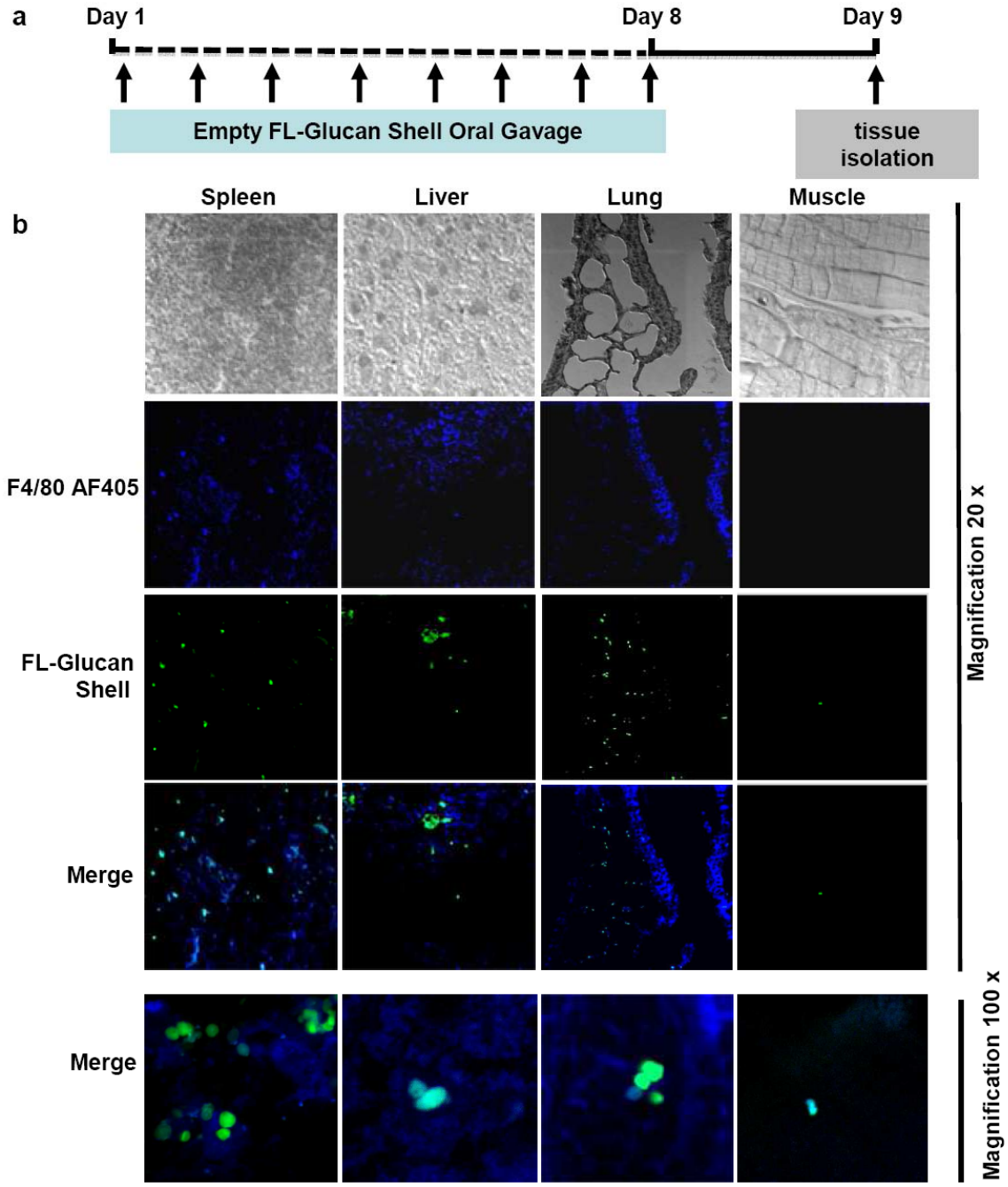
Supplementary Figure 3.1. Model for GeRP uptake by M cells and macrophages in the gut associated lymphatic tissue (GALT). GeRPs in the intestinal lumen enter M cells and undergo transcytosis. As they undergo exocytosis, macrophages in the GALT internalize the GeRPs by phagocytosis. Gastrointestinal macrophages can migrate out of the GALT and travel through the lymphatic and circulatory systems to various tissues such as spleen, liver and lung.



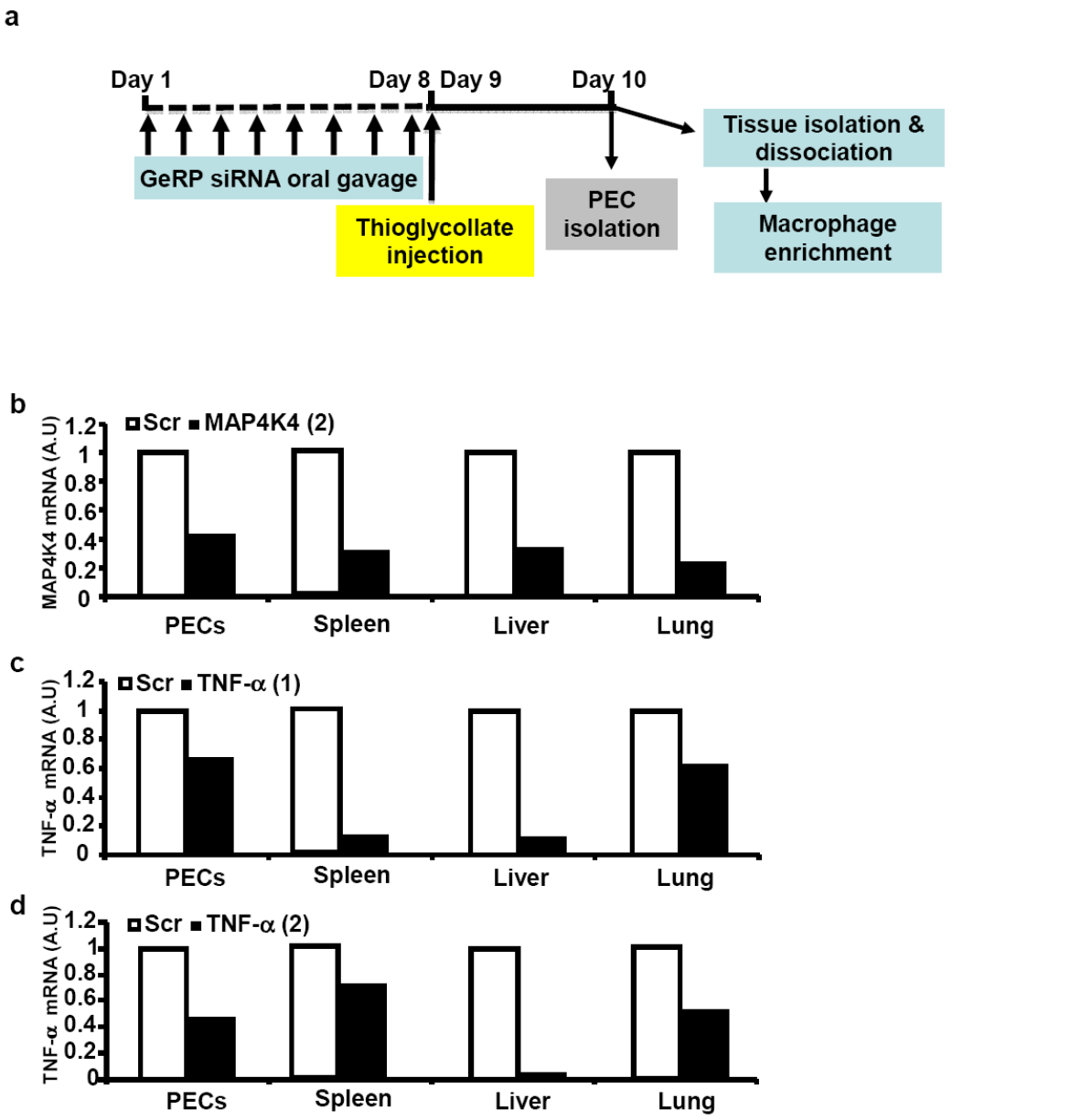
Supplementary Figure 3.2. Gene silencing by a second siRNA targeting MAP4K4 (oligo 2 in Supplementary Table 3.1) and two distinct TNF-α siRNAs (oligos 1 and 2 in Supplementary Table 3.1, respectively. (a) 10^6 PECs were treated with 10^7 GeRPs loaded with 40 pmoles of Scr or a second MAP4K4 siRNA (oligo 2). Total RNA was harvested 48 hours after treatment and analyzed by real time PCR for the expression of MAP4K4 mRNA. (b) LPS-induced TNF-α expression in PECs treated with Scr- or MAP4K4 (oligo 2) siRNA-GeRPs, (c) and (d) LPS-induced TNF-α expression in PECs treated with GeRPs loaded with 40 pmoles of Scr, TNF-α (oligo1) or TNF-α (oligo 2) siRNA. PEC and GeRP preparations were done by Myriam Aouadi and me along with RNA preparation and real time PCR analysis.



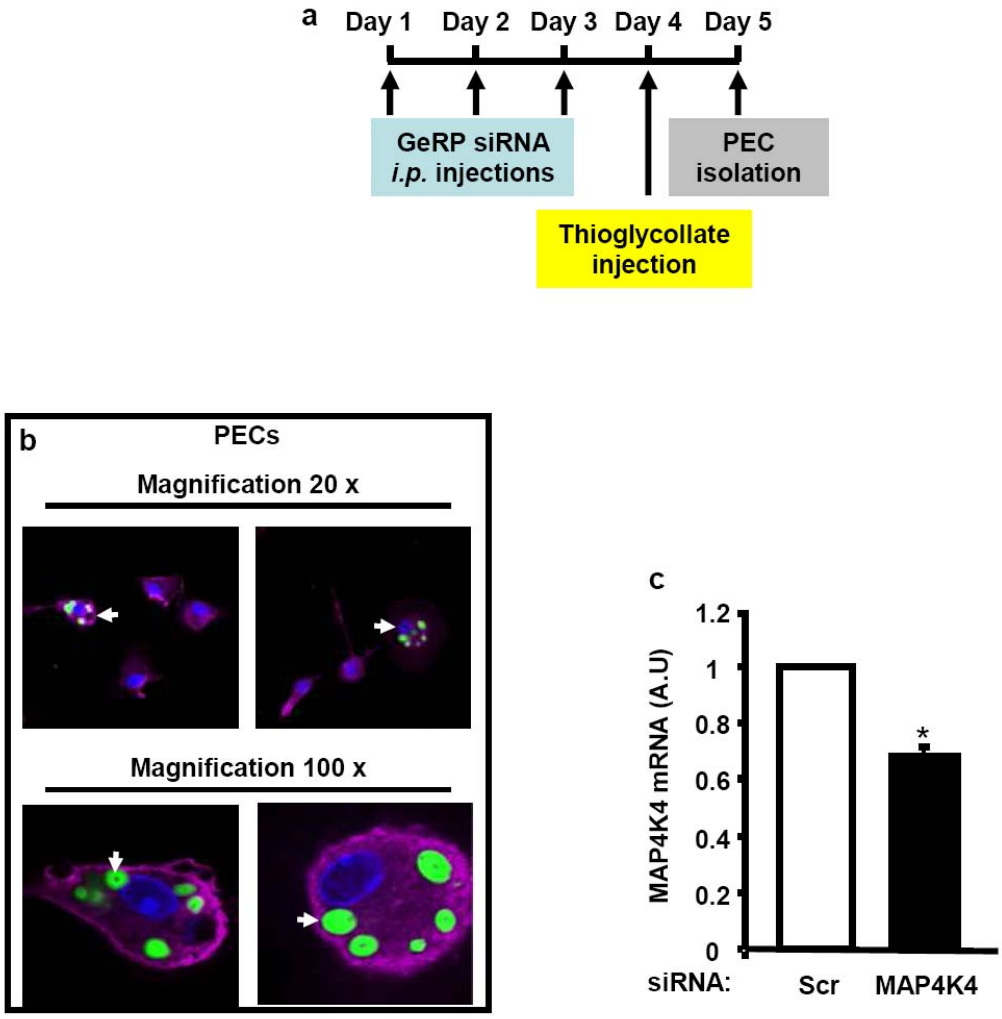
Supplementary Figure 3.3. GeRPs loaded with scrambled siRNA or unloaded GeRPs(which contain tRNA/PEI cores but no siRNA) have no effect on LPS-induced TNF-α secretion. (a) 10^6 PECs were treated with PBS, 10^7 unloaded GeRPs or GeRPs loaded with 40 pmoles of Scr. TNF-α levels in PEC media was measured by ELISA. Myriam Aouadi prepared the GeRPs and I prepared the PECs. ELISA for TNFα was performed by Myriam Aouadi.



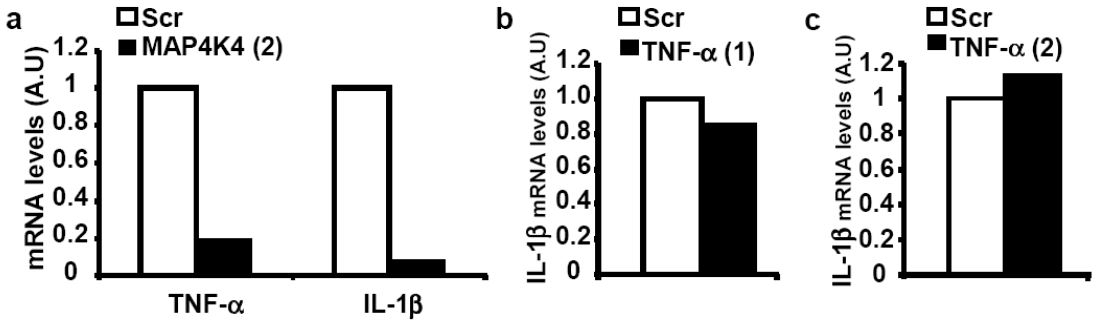
Supplementary Figure 3.4. Orally administered FL-glucan shells are taken up by migratory gut macrophages and traffic into spleen, liver and lung. (a) Timeline of oral administration of FL-glucan shells and tissue collection. (b) Staining with F4/80-AF405 confirmed that spleen, liver and lung macrophages contained FL-glucan shells (green). Upper panels, magnification: 20x and lower panel, magnification: 100x. Myriam Aouadi and I performed all animal handling. Dissections were performed by Myriam Aouadi, Sarah Nicoloro and My Chouinard. Histological staining and image analysis was done by Sarah Nicoloro.



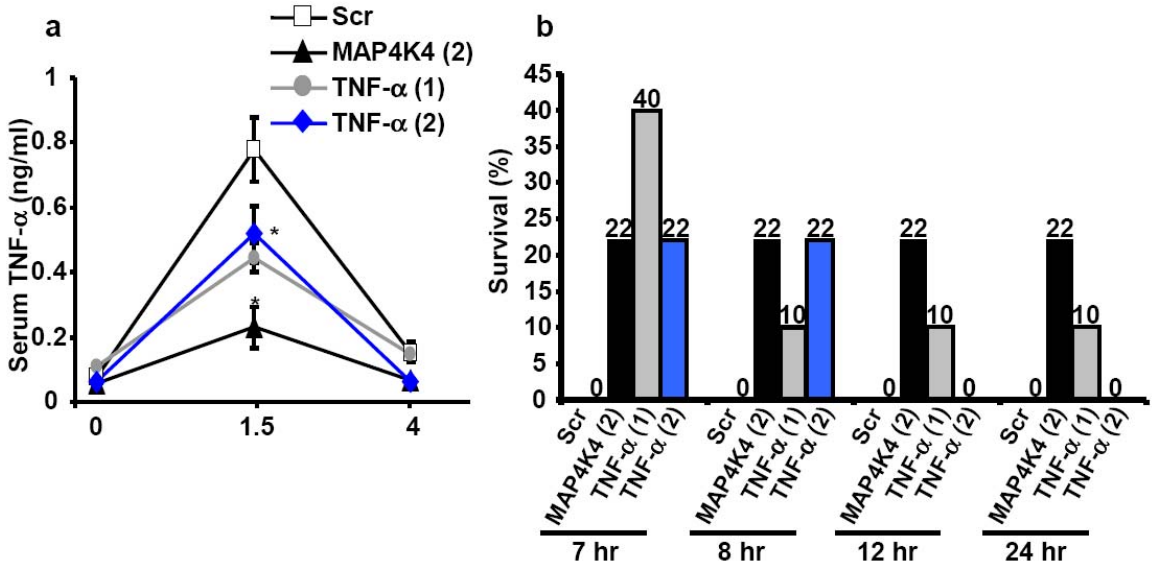
Supplementary Figure 3.5. Orally administered GeRPs containing a second MAP4K4 siRNA or TNF-α siRNA silence MAP4K4 and/or TNF-α expression, respectively, in PECs, and spleen, liver and lung macrophages. (a) Timeline of oral administration of siRNA-GeRPs and tissue collection. Analysis of MAP4K4 or TNF-α expression in PECs and adherent cells isolated from spleen, liver and lung from mice gavaged with GeRPs loaded with 10 ug/kg of Scr, (b) a second siRNA targeting MAP4K4 (oligo 2), or two siRNAs against TNFa (c) TNF-α (oligo 1) or (d) TNF-α (oligo2). GeRP preparations, mouse gavages, and dissections were done by Myriam Aouadi and me. Myriam Aouadi and I did the RNA isolation and real time PCR.



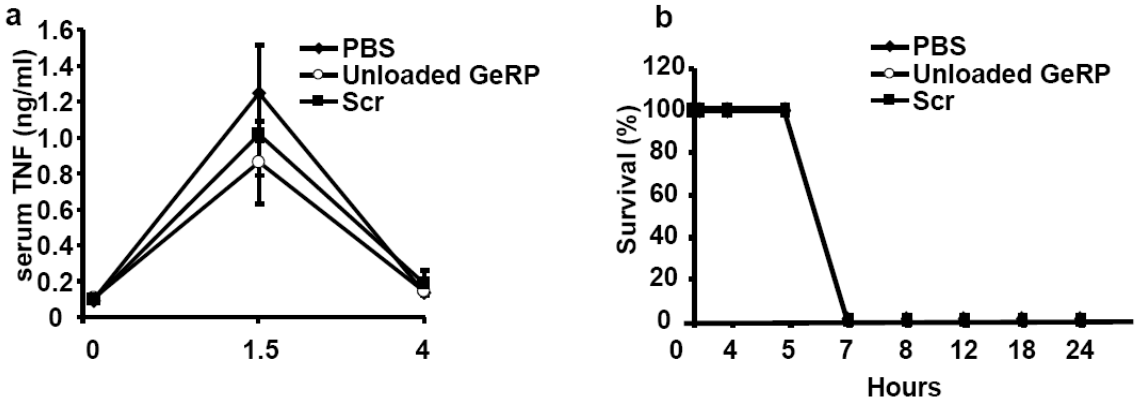
Supplementary Figure 3.6. *i.p.* administration of GeRPs containing MAP4K4 siRNA reduce MAP4K4 mRNA expression in macrophages *in vivo*. (a) Timeline of *i.p.* treatment with siRNA-GeRPs and PEC isolation. (b) Confocal microscopy of PECs. Staining with F4/80-AF633 (Magenta) confirmed that PECs contained GeRPs (green). Nuclei (N) were stained with DAPI (blue). Arrows point to representative GeRPs. Upper panels, magnification: 20x and lower panel, magnification: 100x. (c) MAP4K4 mRNA expression. Results are expressed in arbitrary units and are the mean ± SEM of four independent experiments. Significance was determined using Student's t-test * p <0.001. Animal handling, gavage and dissection were done by Myriam Aouadi and me. Histological staining and image analysis was done by Sarah Nicoloro. RNA isolation and real time PCR was performed Myriam Aouadi and me.



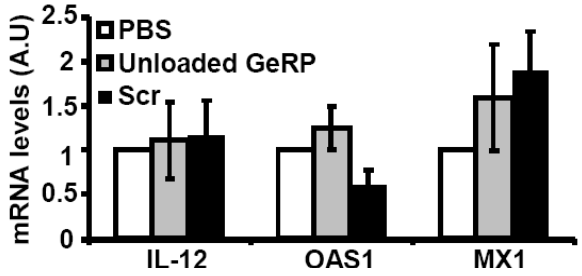
Supplementary Figure 3.7. MAP4K4, but not TNF-α silencing inhibits IL-1β expression. Expression of TNF-α and/or IL-1β in PECs isolated from mice orally treated with GeRPs containing 10 ug/kg of Scr, (a) MAP4K4 (oligo 2), (b) TNF-α (olgio 1) or (c) TNF-α (oligo 2) siRNA. Myriam Aouadi and I did all animal handling and gavages. Myriam Aouadi, Sarah Nicoloro and I dissected all mice. RNA isolation and real time PCR was performed Myriam Aouadi and me.



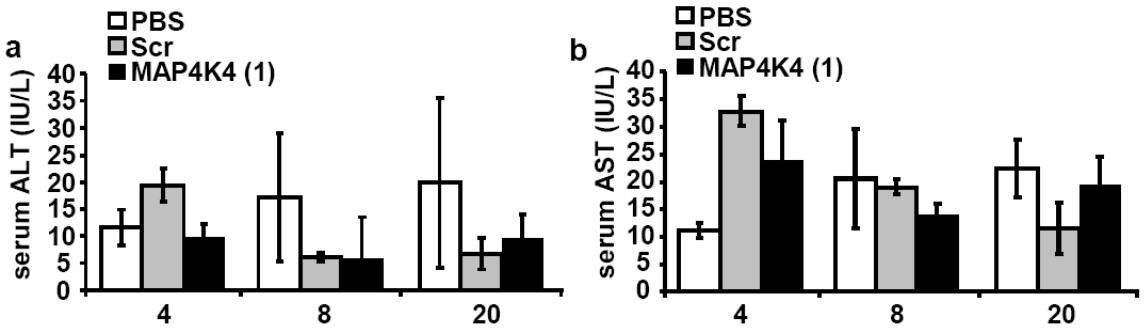
Supplementary Figure 3.8. A second siRNA targeting MAP4K4 (oligo 2 in Supp Table 1) inhibits LPS-induced TNF-α production and lethality *in vivo*. Mice were gavaged with siRNA-GeRPs. Four hours after the final gavage, mice were *i.p.* injected with D-GalN, followed by an *i.p.* injection of LPS. (a) Serum TNF-α levels in siRNA treated mice 1.5 and 4 hours after LPS/D-GalN injection. Results are the mean ± SEM (n=5). Statistical significance was determined by ANOVA and Tukey post test; *$p < 0.05$. (b) Percent survival of mice orally treated with siRNA-GeRPs followed by LPS/D-GalN injections. As the Figure shows, no mice treated with GeRPs containing Scr siRNA survived, even for 7 hours. Myriam Aouadi and I did all animal handling, gavages, injections, bleeding and observations. TNFα ELISA was done by Sarah Nicoloro.



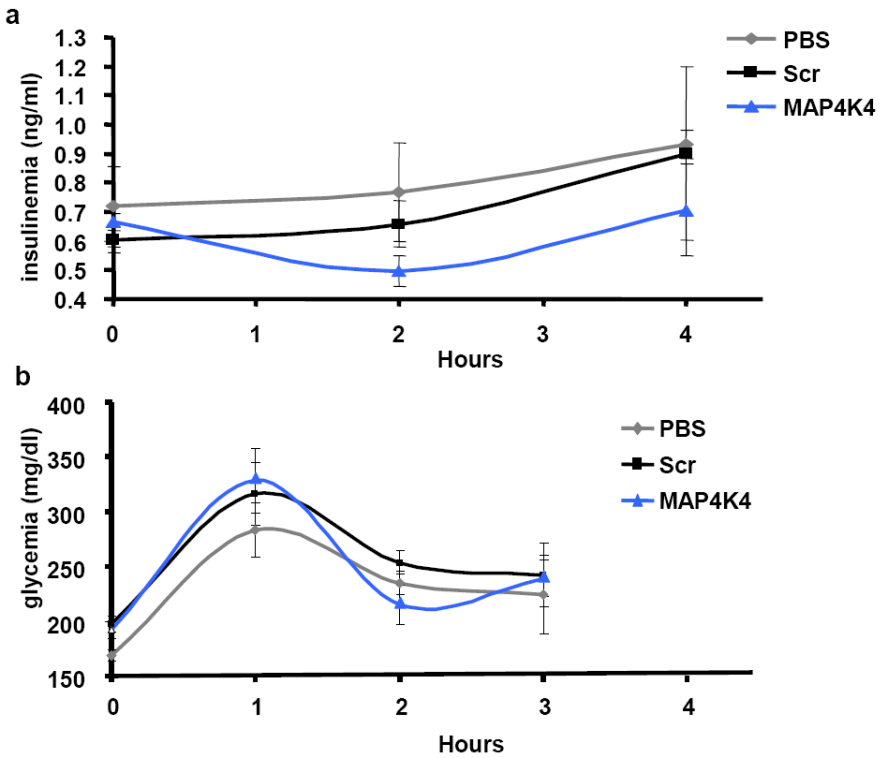
Supplementary Figure 3.9. Unloaded GeRPs (containing tRNA/PEI cores but no siRNA) or GeRPs loaded with scramble siRNA have no effect on LPS-induced TNF-α production and lethality *in vivo.* Mice were gavaged with unloaded GeRPs or GeRPs loaded with 10 ug/kg of Scr siRNA. Four hours after the final gavage, mice were *i.p.* injected with D-GalN, followed by an *i.p.* injection of LPS. (a) Serum TNF-α levels were measured 1.5 and 4 hours after LPS/D-GalN injection. Results are the mean ± SEM (n=5). Statistical significance was determined by ANOVA and Tukey post test. (b) Percent survival of mice orally treated with siRNA-GeRPs, and then injected with LPS/D-GalN. Survival was assessed every hour for 24 hours. Myriam Aouadi and I did all animal handling, gavages, injections, bleeding and observations. TNFα elisa was done by Sarah Nicoloro.



Supplementary Figure 3.10. siRNA-GeRPs failed to elicit an interferon response *in vitro*. 10^6 PECs were treated with PBS, 10^7 unloaded GeRPs(containing tRNA/PEI cores but no siRNA) or GeRPs loaded with 40 pmoles of Scr. Total RNA was harvested 48 hours after treatment and analyzed by real time PCR for the expression of INFβ target genes, OAS1 and MX1 or INFγ target genes, IL-12. Results are the mean ± SEM (n=3). PEC, GeRP and RNA preparation and real time PCR was done by Myriam Aouadi.



Supplementary Figure 3.11. siRNA-GeRP oral treatment fails to alter serum liver enzyme levels *in vivo*. Mice were gavaged with PBS or GeRPs containing 10 ug/kg of Scr or MAP4K4 (1) siRNA. (a) Alanine aminotransferase (ALT) and (b) aspartate aminotransferase (AST) were measured in serum 4, 8 and 20 days after the last gavage (n=3). Animal handling, gavages, bleeding and handling along with AST and ALT assays were done by Myriam Aouadi and me.



Supplementary Figure 3.12. MAP4K4 silencing fails to affect LPS regulation of blood glucose and insulin levels. Mice were gavaged with PBS or GeRPs loaded with 10 ug/kg of Scr or MAP4K4 (oligo 1) siRNA once a day for 8 days. Four hours after the final gavage, mice were *i.p.* injected with D-GalN, followed by an *i.p.* injection of LPS. (a) Serum insulin levels 2 and 4 hours after LPS/D-GalN injection. (b) Serum glucose levels 1, 2 and 3 hours after LPS/D-GalN injection. Results are the mean ± SEM (n=5). Statistical significance was determined by ANOVA and Tukey post test. Animal handling, gavages, bleeding, injections and handling along with AST and ALT assays were done by Myriam Aouadi and me.

	Accession numbers	siRNA sequence	Percentage *in vitro* knockdown	Percentage *in vivo* knockdown
Scramble	–	5'-CAGUCGCGUUUGCGACUGG-3'	0	0
Map4K4 (1)	NM_008696	5'-GACCAACUCUGGCUUGUUA-3'	72	70
Map4K4 (2)		5'-CAGAAGTGGCCAAGGGAAA-3'	60	60
TNF-α (1)	NM_013693	5'-CUGUUGGUUGAUCACCACG-3'	40	33
TNF-α (2)		5'-GCATGGATCTCAAAGACAA-3'	31	54

Supplementary Table 3.1. Observed *in vitro* and *in vivo* (oral delivery of GeRPs) knockdown with various siRNA oligonucleotides in GeRPs. The data in columns at right represent the percent decreases in cognate gene expression observed due to the respective siRNA in GeRPs in our studies.

Hours after *LPS/D-Galactosamine Injection*	*Number of Mice Surviving at Indicated Time Point*		
	PBS	Scr	MAP4K4
0	11/11	22/22	22/22
6	4/11	14/22	18/22
7	1/11	3/22	15/22
8	1/11	2/22	11/22
10	1/11	2/22	9/22
24	1/11	2/22	8/22

Supplementary Table 3.2. Survival post LPS-challenge of mice treated with PBS, Scr or MAP4K4 (1) siRNA-GeRPs.

	Chi-Square	Df	Sig.
Log Rank	8.334	1	0.004
Breslow	8.799	1	0.003
Tarone-Ware	8.99	1	0.003

Supplementary Table 3.3. Statistical testing of the equality of survival probabilities between Scr and MAP4K4 (1) siRNA-GeRP treatments in the LPS/D-galactosamine challenge.

Primer	Sequence
36B4 F	GACCATTAGCCTTGTGTGTACTGTATG
36B4 R	TGGATCGATTGTGCTTCAAGTT
MAP4K4 F	CATCTCCAGGGAAATCCTCAGG
MAP4K4 R	TTCTGTAGTCGTAAGTGGCGTCTG
TNF-α F	CCCTCACACTCAGATCATCTTCT
TNF- α R	GCTACGACGTGGGCTACAG
IL-1β F	GCAACTGTTCCTGAACTCAACT
IL-1 β R	ATCTTTTGGGGTCCGTCAACT
IL-10 F	CTGGACAACATACTGCTAACCG
IL-10 R	GGGCATCACTTCTACCAGGTAA
CCR2-F	ATCCACGGCATACTATCAAGATC
CCR2-R	CAAGGGTCACCATCATGGTAG
OAS1-F	ATTACCTCCTTCCCGACACC
OAS1-R	CAAACTCCACCTCCTGATGC
MX1-F	GATCCGACTTCACTTCCAGATGG
MX1-R	CATCTCAGTGGTAGTCAACCC
IL-12p40	AGACATGGAGTCATAGGCTCTG
IL-12p40	CCATTTTCCTTCTTGTGGAGCA

Supplementary Table 3.4 Primer sequences

Discussion

Several technical features of the GeRP delivery system described here are notable. Most remarkable, the *in vivo* potency of 10 μg siRNA/kg in GeRPs to mediate gene silencing is 10 to 500 times greater than previous studies reporting systemic delivery by intravenous injection. For significant effects *in vivo*, intravenous injection of siRNA formulations require doses ranging from 125 μg/kg to 50 mg/kg in mice[246-251] and 1 mg/kg in nonhuman primates[252]. For attenuation of LPS-induced lethality in mice by *i.p.* injection, 1.2 mg TNF-α siRNA/kg was required[344]. These studies generally employed chemically modified siRNA to enhance stability. The high potency of orally delivered siRNA within GeRPs (10 μg/kg) is all the more surprising since unmodified siRNA was used in our studies. This high potency is likely due to protection of siRNA against nuclease degradation by PEI within GeRPs, low nonspecific binding of the GeRPs enroute to Peyers' patches of the gut, and to the high efficiency of GeRP uptake by phagocytic cells in the GALT. Furthermore, the siRNA loading capacity within the hollow cavity of glucan shells is far greater than we have used here, and has the potential to orally co-deliver combinations of siRNA, DNA, proteins and small molecules. It should also be noted that while we have focused our attention on macrophages in this study, we cannot rule out the possibility that other cell types are also targeted by oral delivery of GeRPs.

The present results demonstrate a potent attenuation of the macrophage inflammatory response to LPS following GeRP-mediated delivery of siRNA against TNF-α (Supplementary Figs. 3.2 and 3.8) or MAP4K4 (Figs. 3.2, 3.5 and

Supplementary Figs 3.2 and 3.8) have significant therapeutic implications. Inflammatory cytokines and TNF-α in particular are pathogenic in humans, and injectable anti- TNF-α protein therapeutics are successful commercial products for the treatment of rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and psoriasis[345]. Macrophage-mediated pathogenesis is also well characterized in mouse models of obesity-associated insulin resistance[346] and atherosclerosis[347], while such autoimmune diseases as Type 1 Diabetes involve the deleterious actions of inflammatory cytokines[348,349]. Further development of GeRP-mediated delivery of siRNA to attenuate inflammation for these and other human maladies will be a major focus of our future studies.

Limitations and Future Perspectives

The results presented in this chapter are exciting for therapeutic reasons. However, numerous interesting questions remain and need to be addressed to further this technology. One immediate question that we are currently addressing is why the level of knockdown observed is greater than the amount of macrophages containing GeRPs. It is possible that the GeRPs are partially degraded by oral delivery and that the fluorescent tag is lost despite maintaining the ability to silence gene expression. Thus, studies are required to determine the durability of GeRPs in the fluids and enzymes of the gastrointestinal system. We also plan to utilize different chemical linkages and brighter fluorescent tags that may better survive oral delivery and allow for easier visualization *in vivo*. Additionally, knockdown may persist despite the presence of low amounts of GeRP containing macrophages because the siRNA may also transfer to other macrophages. This may occur through direct cellular contact or through the

exocytosis of partial siRNA containing GeRPs which are phagocytosed by other macrophages. Experiments are underway to determine if exocytosed materials from GeRP treated macrophages can mediate gene silencing in untreated macrophages.

An additional area of investigation remaining is the mechanism by which MAP4K4 regulates the production of TNFα in response to LPS. Our results suggest that the LPS activation of MAPK and NFκB signaling pathways remains intact following MAP4K4 silencing. Thus, a novel role for the MAP4K4 regulation of TNFα production may exist. One possibility is PPARγ. As previously mentioned, MAP4K4 negatively regulates PPARγ expression in adipocytes. Recent studies have demonstrated that PPARγ can suppress inflammatory gene expression[350] and that this function is required in macrophages to maintain insulin sensitivity[351,352]. Hence, it is plausible that MAP4K4 functions to suppress PPARγ in macrophages, as it does in adipocytes. We also want to determine if MAP4K4 suppresses macrophage TNFα production in response to other TLR and general inflammatory agents. Perhaps MAP4K4 may regulate TNFα production in obesity.

Finally, we plan to test the ability of GeRPs to transfer siRNA to other non-macrophage cell types. Through conjugation of GeRPs with antibodies recognizing cell type specific markers GeRPs could potentially deliver siRNA to additional cell types. Although macrophages are an important drug target, gene silencing in other cell types is also of immense benefit in fighting other diseases. By answering these questions, we may achieve better insight into the regulation of TNFα production by MAP4K4 and better utilization of the GeRP technology.

CHAPTER IV: Discussion

A major underlying mechanism leading to insulin resistance is the dysfunction of adipocytes and macrophages. As mentioned earlier, adipose tissue is essential in maintaining systemic glucose homeostasis as the complete absence of adipose tissue causes insulin resistance in both humans and mice[222-227]. Impairing adipose function, through the selective loss of GLUT4 in adipocytes, causes systemic insulin resistance[29]. Conversely, enhancing adipose function, through adipocyte specific GLUT4 over expression, causes enhanced insulin sensitivity and protects against high fat diet induced insulin resistance[26]. Additionally, inflammation from macrophages causes insulin resistance in mouse models[346]. Thus, the goals outlined for this thesis were to better understand the role of MAP4K4 in adipocyte and macrophage inflammatory processes.

Previously our lab demonstrated that the germinal center kinase MAP4K4 negatively regulates PPARγ expression and triglyceride synthesis in adipocytes[275]. The authors also showed that MAP4K4 mediated part of the negative effects of TNFα on adipocyte gene expression. Considering that enhancements of adipocyte function are of therapeutic interest, a better understanding of the signaling pathways utilized by TNFα to increase MAP4K4 expression was needed. Thus, in Chapter II, I defined a pathway by which TNFα increases MAP4K4 expression. Also, earlier studies had implicated MAP4K4 as a potential mediator of TNFα production in lymphocytes[277]. Because of the importance of macrophages in driving the inflammatory response in multiple diseases including insulin resistance, I wanted to determine if MAP4K4

controlled TNFα production in macrophages *in vivo*. Thus, in Chapter III I demonstrated that MAP4K4 also regulates macrophage TNFα production in response to LPS *in vivo*. To achieve *in vivo* macrophage gene silencing, the GeRP technology was developed. Together, these studies better characterizes the role of MAP4K4 in adipocyte and macrophage inflammation.

The Regulation of MAP4K4 Expression by TNFα in Adipocytes

In Chapter II of this study, the regulation of MAP4K4 by TNFα in adipocytes was characterized. This study was important for determining the mechanisms by which TNFα regulates adipocyte biology through MAP4K4. As mentioned earlier, MAP4K4 was previously demonstrated to negatively regulate PPARγ and lipogenic gene expression[275]. The role of TNFα as a negative regulator of adipocyte gene expression had been realized for a number of years[122,123]. However, the majority of these studies attributed most of the effects of TNFα on gene expression to NFκB. Thus, the discovery that silencing of MAP4K4 prevents the full attenuation of adipocyte genes by TNFα suggested a potential role for MAP4K4 in the negative regulation of adipocyte function by TNFα[275]. Hence, by understanding how TNFα regulated MAP4K4 expression, new insight into the mechanisms by which TNFα regulates adipocyte function was gained through these studies.

An important finding in these studies was that cJUN and ATF2 controlled MAP4K4 expression in response to TNFα. Figure 2.6A demonstrated that silencing of either gene attenuates the TNFα mediated increase in MAP4K4 expression. As a positive control IL-6 was used since it is robustly upregulated in response to TNFα

treatment. Confirming earlier studies, I showed that increases in IL-6 transcript levels did not require cJUN or ATF2 (Figure 2.6B) [306,307]. In these studies, the authors demonstrated that FRA-1 and JUND, but not cJUN or ATF2, bind AP-1 sequences within the IL-6 promoter. This result confirmed the specificity of the pathway hypothesized as other TNFα induced transcripts, like IL-6, did not appear to be altered. However, the possibility of other transcription factors regulated by TNFα mediating the increase in MAP4K4 expression cannot be ruled out from these studies. Thus, in the future, determining the role NFκB or other transcription factors play would be of interest.

The requirement of TNFR1, but not TNFR2 for the effect of TNFα on MAP4K4 expression was consistent with the literature regarding TNFα signaling in adipocytes (Figure 2.7A and 2.7B). Most of the TNFα effects on adipocytes have been attributed to TNFR1, not TNFR2. In fact, signaling through TNFR1, but not TNFR2 increases lipolysis in 3T3-L1 Adipocytes[124]. Furthermore, the authors went on to demonstrate that the negative effects of TNFα on insulin signaling are also through TNFR1, but not TNFR2, suggesting that TNFR2 is not an important component of TNFα signaling in adipocytes. However, we detected a more subtle role for TNFR2 in the regulation of IL-6. Unlike MAP4K4, which did not require TNFR2 for full induction, the TNFα mediated increase in IL-6 expression did require TNFR2 signaling for the full effect. This result demonstrated the specificity of our system and that the TNFα effect on MAP4K4 expression was through TNFR1 but not TNFR2.

Surprisingly, not all cytokines or inflammatory stimuli tested were capable of inducing MAP4K4 expression. It is interesting to note that although numerous cytokines

can impair insulin sensitivity, each cytokine appears to use a unique mechanism. IL-1β and TNFα are excellent examples of this point. Both cytokines are potent mediators of inflammation that are elevated in obese humans[127,128]. Additionally, both cytokines are capable of suppressing IRS-1 phosphorylation and attenuating insulin signaling[113,115,116,129,130]. However, unlike TNFα which has a potent negative effect on lipogenic genes[122,123], IL-1β treatment fails to alter the adipogenic genes PPARγ and C/EBPα[129,130]. Interestingly, in our studies, IL-1β also failed to increase MAP4K4 expression (Figure 2.3B), despite being in excess of concentrations required to activate IL-6 mRNA expression (Figure 2.3C). Thus, the data presented in this body of work is consistent with the idea that TNFα is a unique cytokine because of its ability to influence adipocyte gene expression through the activation of NFκB and through MAP4K4.

The Development of Technology for in vivo Delivery of siRNA

For the second part of this thesis, the role of MAP4K4 in macrophages was analyzed *in vivo*. Thus, a major component of these studies was the development and characterization of technology to analyze the role of macrophage MAP4K4 *in vivo* with siRNA. The first major obstacle in the usage of siRNA *in vivo* is tissue specificity. To date, the majority of *in vivo* siRNA studies have failed to achieve tissue specificity due to the methods utilized. For example, the so called “high pressure” transfection technique, in which siRNAs is injected into the tail vein, primarily targets hepatocytes[246,353]. Although this model has succeeded in attenuating Hepititis C infenctions *in vivo*, this transfection method may not be suitable for use in humans[246]. Therefore, more specific *in vivo* transfection methods are needed.

Other studies have also demonstrated limited success in terms of *in vivo* siRNA delivery to primary lymphocytes. These cells are difficult to target for a number of reasons. First, they do not readily transfect with conventional reagents such as lipofectamine or oligofectamine[248,354]. Secondly, cells of the immune system typically broadly distribute and fail to localize simply to one part of the body. Thus, a highly efficient systemic delivery method is needed. One approach succeeded in targeting siRNA to cells expressing HIV-1 envelope protein, including the difficult-to-transfect primary T cells, by utilizing siRNAs complexed with antibodies against HIV-1 envelope protein[249]. Other studies utilizing antibodies against lymphocyte markers have also demonstrated success in attenuating lymphocyte mediated colitis[248,355]. Although these approaches do look promising, they are limited to tissue specific antigens that may or may not exist on target cells, and high quality antibodies which may not be attainable. Thus, an orally deliverable mechanism capable of systemic delivery to specific cell types would be of immense clinical interest.

Usage of GeRPs for the delivery of RNAi allows for tissue specificity with a common dietary component of yeasts. Remarkably, β1,3-D-glucan is transcytosed in the intestine through M-cells to the GALT where macrophages readily phagocytose the particles (Supplemental Figure 3.1)[322-325]. The specific phagocytosis by macrophages is mediated in part by the Dectin-1 receptor. Because macrophages and monocytes are the primary phagocytes expressing the receptor, siRNA encapsulated within GeRPs target only these cell types[324,325]. In support of this hypothesis, gene silencing was observed in multiple tissue macrophage populations including, peritoneal, spleen, lung and liver (Figure 3.4C). In tissues, such as skeletal muscle, where low macrophage

populations are observed, gene silencing was not detected suggesting the specificity of the macrophage targeting (Figure 3.4C). Microscopy images from these studies further illustrated the specificity as GeRPs were detected in macrophages isolated from the tissues where silencing was readily observed (Figure. 3.4A and Supplemental Figure 3.4). Altogether, the results presented in Chapter III suggest that the oral delivery of siRNA in GeRPs can target macrophages systemically.

A second major obstacle in the therapeutic use of siRNA is the potential toxicity of siRNA. The initial excitement regarding the potential therapeutic usage of siRNA was dampened by the discovery that naked siRNA can induce a powerful interferon response in cultured cells[318,356]. More recently, the beneficial effects of RNAi therapeutics in mouse models of choroidal neovascularization, were demonstrated to be non-specific effects caused by TLR3 mediated production of IFNγ and IL-12. Interestingly, in these studies both pro- and anti-angiogenic siRNA targets all suppressed choroidal neovascularization[357]. Unlike the systems utilized in these earlier studies, activation of the interferon response system is not observed utilizing GeRPs. As demonstrated in Supplementary Figure 3.10, interferon response gene expression was unaffected by GeRP treatment. Furthermore, after 8 days of GeRP treatment *in vivo*, serum TNFα (Supplemental Figure 3.9A) and IFNγ (Figure 3.4D) levels were unaltered. Furthermore, serum AST and ALT liver enzyme levels were all within normal ranges following GeRP treatment suggesting that there are no long term liver toxicity issues as observed nonhuman primates treated with RNAi via the stable nucleic acid lipid particle technology[252].

Because β1,3-D-glucan shells are common components of bakers yeasts, humans have been readily consuming the β1,3-D-glucan shells for millennia. Therefore, issues regarding the safety of glucan as a drug delivery system are not anticipated. In agreement, we did not detect an increase in TNFα serum levels following more than 10 days of glucan shell treatment (Figure 3.5B). Hence, it is likely that the glucan shell, in which the siRNA is encapsulated, protects the siRNA from detection until phagocytosis, thus preventing the activation of the interferon or other cytokine responses.

MAP4K4 Regulation of Macrophage LPS Stimulated TNFα Production

By utilizing the GeRP system the role of MAP4K4 *in vitro* and *in vivo* was analyzed. An important discovery in these studies was that MAP4K4 regulated macrophage LPS stimulated TNFα production. Silencing of MAP4K4 inhibited LPS stimulated TNFα mRNA by 50% and protein by 30% in primary macrophages cultured *in vitro* (Figures 3.2D 3.2E respectively). A detailed analysis of the signaling cascades activated by LPS signaling in macrophages demonstrated that phosphorylation of the major signaling cascades remained intact (Figure 3.3).

IL-1β was another cytokine observed to be positively regulated by MAP4K4 (Figure 3.5C). As mentioned earlier, IL-1β and TNFα can act synergistically in models of inflammation[339]. However, other macrophage markers, such as the MCP-1 receptor (CCR2) or IL-10 failed to change significantly upon MAP4K4 silencing (Figure 3.5C), suggesting that MAP4K4 specifically regulates a select group of cytokines. An area of

interest for the future would be a more comprehensive analysis of cytokine expression profiles of macrophages in which MAP4K4 has been silenced.

Keystone to these studies was the demonstration that GeRPs can silence MAP4K4 expression and attenuate LPS mediated inflammation *in vivo*. Previous studies had demonstrated that attenuation of TNFα and IL-1β release protected mice from the lethal effects of LPS[341,342]. Thus, we tested MAP4K4 silencing in the LPS/D-GalN model and observed increased survivability upon MAP4K4 silencing (Figure 3.6A and Supplementary Figure 3.8B). However, previous reports suggested that anti TNFα siRNA administration alone prevented death in mice challenged with LPS by 75%[344]. In this study, a single injection of siRNA against TNFα lowered TNFα protein levels by almost 80%. Therefore in Chapter III, we also test survivability of mice treated with GeRPs containing siRNA against TNFα. In our hands, two different oligos potently silenced TNFα expression (Supplemental Figures 3.2C and 3.2D), and attenuated the increase in serum TNFα during the LPS D-GalN challenge (Supplemental Figure 3.8A). Despite the success of these oligos, enhanced survival was not observed following LPS/D-GalN treatment (Supplemental Figure 3.8B). Instead, a slight delay in the onset of death was observed. Whether or not this delay was due to the effect of TNFα is an area of future interest.

One potential explanation for why TNFα silencing was not as efficient as previously reported, is that our silencing was not as complete as the silencing observed in these earlier studies[344]. In these previous studies mice were injected with an enormous amount of siRNA (1.2 mg/kg) when compared to our methods (10 μg/kg). A,

second potential explanation for this outcome is that the injection of copious anti TNFα siRNA silenced TNFα expression in additional cell types that respond to LPS and secrete TNFα, such as T-cells[343]. It is likely that TNFα attenuation does not occur in T-cells with GeRPs. Furthermore, IL-1β levels were not changed by TNFα silencing (Supplemental Figure 3.7B and 3.7C) as they are by MAP4K4 silencing (Figure 3.5C). Thus, the increase in IL-1β following LPS/D-GalN stimulation may be sufficient to cause death with TNFα silencing. However, silencing MAP4K4 reduces the expression of both TNFα and IL-1β which may explain why MAP4K4 silencing protects better. Interestingly, the decrease in IL-1β was also observed with the use of a second oligo targeting MAP4K4 (Supplemental Figure 3. 7A). This data suggests that the decrease in both TNFα and IL-1β following MAP4K4 silencing is a specific effect and potentially necessary for protection in the LPS/D-GalN model system.

Future Perspectives

While small molecular inhibitors remain difficult to design, this study demonstrates that tissue specific depletion of MAP4K4 and any other macrophage target with RNAi is possible through GeRP technology. We do not know if the GeRP technology silences gene expression in other tissue types, such as adipocytes or hepatocytes. However, gene silencing in macrophages alone is a remarkable achievement as macrophages mediate numerous clinical diseases including atherosclerosis[347] and obesity-associated insulin resistance[346].

In the future, the GeRP technology will be used to test the role of macrophage MAP4K4 function in mouse models of insulin resistance. As of now no data exists to suggest that macrophage MAP4K4 regulates obesity related inflammation. If MAP4K4

also positively regulates macrophage TNFα expression in mice fed high fat diets, the GeRPs may potentially improve insulin sensitivity in these models. Because of the role of MAP4K4 in negatively regulating adipocyte function, MAP4K4 may represent a pleiotropic target for attenuating insulin resistance. In agreement, the attenuation of MAP4K4 in adipocytes enhances triglyceride storage[275]. Treatments, such as thiazolidinediones, which enhance adipose tissue triglyceride storage improve insulin sensitivity[232]. Thus, the production of the inflammatory cytokines TNFα and IL-1β from macrophages, and the release of FFA from adipose tissue may both be regulated by MAP4K4 in obesity. We admit to not knowing the physiological role of macrophage MAP4K4 in the development of insulin resistance. However, given the link between MAP4K4 and LPS induced TNFα expression discovered in Chapter III, we plan to test the function of macrophage MAP4K4 expression rigorously in mouse models of insulin resistance.

Additionally, in these studies the mechanism by which TNFα controls MAP4K4 expression in adipocytes was characterized in Chapter II of these studies. Although ATF-2 and cJUN are important for the increase in MAP4K4 expression following TNFα treatment, undoubtedly other transcription factors also regulate MAP4K4 expression. One candidate that we plan to test is NFκB which is known to regulate the expression of numerous inflammatory genes. Through experiments such as chromatin immuno-precipitations, we plan to show a direct interaction of ATF-2 and cJUN on the MAP4K4 promoter following TNFα treatment. We would not be surprised if we also observe NFκB binding on the MAP4K4 promoter in these conditions. Thus, a more careful analysis of the MAP4K4 promoter region is a worthwhile study.

Despite the identification of MAP4K4 as a mediator of LPS stimulated TNFα production in macrophages, work is required to determine the mechanism by which this is achieved. First, MAP4K4 may regulate LPS induced TNFα production through a unique signaling mechanism. Identifying interaction partners and signaling components in response to LPS stimulation is of interest. Secondly, it is possible that MAP4K4 silencing may alter LPS stimulated TNFα production by modulating of PPARγ. Indeed, PPARγ deletion in macrophages results in the conversion of macrophages to a highly inflammatory state which exacerbates insulin resistance[352]. Considering that that MAP4K4 negatively regulates PPARγ expression in adipocytes, it is plausible that MAP4K4 functions similarly to suppress PPARγ in macrophages. Furthermore, the role of MAP4K4 in macrophage TNFα production may not be cell type exclusive, as MAP4K4 is also required for optimal TNFα production in T-cells[277]. Thus, does MAP4K4 also regulate TNFα production in adipocytes, a known producer of TNFα during obesity? All of these areas are of interest for future studies regarding MAP4K4.

Lastly, the GeRP technology developed here is not limited to MAP4K4 targeting siRNAs as any expressed macrophage gene can be targeted. This was demonstrated by our targeting of TNFα in Chapter III. The development and characterization of this technology was perhaps the most important contribution of this body of work as the GeRP technology is applicable to any gene expressed in a macrophages. Considering the vast amount of diseases mediated by macrophages, the GeRP technology is full of promise for future clinical application.

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