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Regulation of macrophage inflammatory signaling pathways by AMP-activated protein kinase.

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REGULATION OF MACROPHAGE INFLAMMATORY SIGNALING PATHWAYS BY AMP-ACTIVATED PROTEIN KINASE

By

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A Dissertation Approved on
April 25, 2014

by the following Dissertation Committee:

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Jill Suttles

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DEDICATION

Dedicated to my beloved family and friends.

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ABSTRACT

REGULATION OF MACROPHAGE INFLAMMATORY SIGNALING PATHWAYS BY AMP-ACTIVATED PROTEIN KINASE

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AMP-activated protein kinase, AMPK, is a conserved serine/threonine kinase with a critical function in the regulation of metabolic pathways in eukaryotic cells. Recently, AMPK has been shown to play an additional role as a regulator of inflammatory activity in leukocytes. Treatment of macrophages with chemical AMPK activators, or forced expression of a constitutively active form of AMPK, results in polarization to an antiinflammatory phenotype. Additionally, we reported previously that stimulation of macrophages with antiinflammatory cytokines such as IL-10, IL-4 and TGF-β results in rapid activation of AMPK, suggesting that AMPK contributes to the suppressive function of these cytokines. In the current study we investigated the role of AMPK in the regulation of macrophage antiatherogenic functions and demonstrated a new mechanism for AMPK's role in mediating IL-10-induced gene expression and antiinflammatory effects. The expression of dominant negative (DN-) AMPKα1 in macrophages resulted in a spontaneous obese phenotype in mice associated with fatty

liver and heart enlargement. The expression of constitutively active (CA-) AMPKα1 in macrophages resulted in decreased IL-6 production but increased expression of ABCA1 and ApoE. Compared to wild-type macrophages, AMPKα1-deficient macrophages failed to express atheroprotective genes including ApoE, LXRα, and ABCA1 in response to IL-10. Mechanistic studies revealed that IL-10-stimulated wild-type macrophages displayed rapid activation of PI3K and its downstream target Akt, an effect that was not seen in macrophages generated from AMPKα1-deficient mice. Treatment with the PI3K inhibitor LY294002 blocked IL-10's ability to induce Akt activation but not AMPK activation, suggesting that IL-10-mediated activation of AMPK is independent of PI3K. CA-AMPKα1 macrophages displayed elevated PI3K and CREB activation in response to IL-10 compared to the empty vector transfected macrophages. IL-10 stimulation resulted in increased mTORC1 activity, an Akt downstream target, an effect that was reduced in AMPKα1-deficient mice. IL-10 induced phosphorylation of both Tyr705 and Ser727 residues of STAT3 in an AMPKα1-dependent manner, and these phosphorylation events were blocked by inhibition of CaMKKβ, an upstream activator of AMPK, and by the mTORC1 inhibitor rapamycin, respectively. The impaired STAT3 phosphorylation in response to IL-10 observed in AMPKα1-deficient macrophages was accompanied by reduced SOCS3 expression and an inadequacy of IL-10 to suppress LPS-induced proinflammatory cytokine production. Overall, our data demonstrate that AMPKα1 is required for IL-10 activation of the PI3K/Akt/mTORC1 and STAT3/SOCS3 antiinflammatory pathways regulating macrophage polarization, a mechanism with broad-reaching applicability in immune homeostasis and in inflammation-associated diseases such as atherosclerosis, autoimmune diseases, and obesity.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

FIGURE PAGE

TABLES PAGE

CHAPTER 1

INTRODUCTION

Macrophages are important players of the innate immune system. They display profound phenotypic and functional heterogeneity due to their ability to adapt to the tissue microenvironment (1). A variety of mediators in the microenvironment including type 1 cytokines such as IFNγ and IL-12 (2, 3), or type 2 cytokines such as IL-4, IL-10 and TGFβ (4-7) are able to promote macrophage development and activation toward pro- or anti- inflammatory functional patterns. The changes of the cytokine profile in the microenvironment induce macrophage subsequently shift their functional phenotypes, and display highly balanced patterns of inflammatory features (7, 8). The AMP-activated protein kinase (AMPK) has been well established as a regulator of energy homeostasis in eukaryotic cells and recent research suggests an important role of AMPK as a counter-regulator in many inflammatory processes (9-13). Our previous studies have shown that AMPK responds to either pro- or anti- inflammatory stimuli and plays a key regulatory role in macrophage and dendritic cell (DC) inflammatory functions (14, 15). Changes of macrophage inflammatory functions contribute greatly to the progression or regression of atherosclerosis (16, 17). Many AMPK-regulated metabolic and inflammatory signaling pathways are reported to be protective in atherosclerosis (18, 19). However, the role of macrophage-expressed AMPK in the regulation of atherosclerosis is unclear.

MACROPHAGE FUNCTIONAL PLASTICITY

Macrophage development and heterogeneity

The mononuclear phagocyte system comprises monocytes, macrophages, and their lineage-committed precursors (20). The monocyte precursors are generated from hematopoietic stem cells in the bone marrow, and then monocytes exit the bone marrow, circulate in the blood, and can enter tissues under inflammatory conditions. They give rise to subsets of macrophages and inflammatory DCs (21). Macrophages are phagocytic leukocytes that are present in all tissues of the body. According to the specific tissues that macrophages reside in, they are given unique names such as microglia (central nervous system, CNS), osteoclasts (bone), alveolar macrophages (lung), Langerhans cells (skin), Kupffer cells (liver), *etc*. (22).

Macrophages display extremely plastic characteristics to reversibly adapt to the changes in the microenvironment and subsequently modulate both innate and adaptive immune responses (1, 7, 8). Depending on what agents they encounter in the blood, circulating macrophage precursors enter different tissues where they are influenced by the tissue-specific microenvironment, resulting in alterations of their maturation and development of tissue specific function pattern. A large spectrum of microenvironment factors has been shown to induce macrophage activation and acquisition of functional phenotypes. These factors include bacterial moieties, antibody immune complexes, cytokines, chemokines, fatty acids, and stress hormones (7, 22, 23). Stimulation by one factor alone, or in combination, results in macrophage performance of a wide range of activities, many are opposite to one another (7). The variety of macrophage activities participates and plays regulatory roles in many important biological processes including

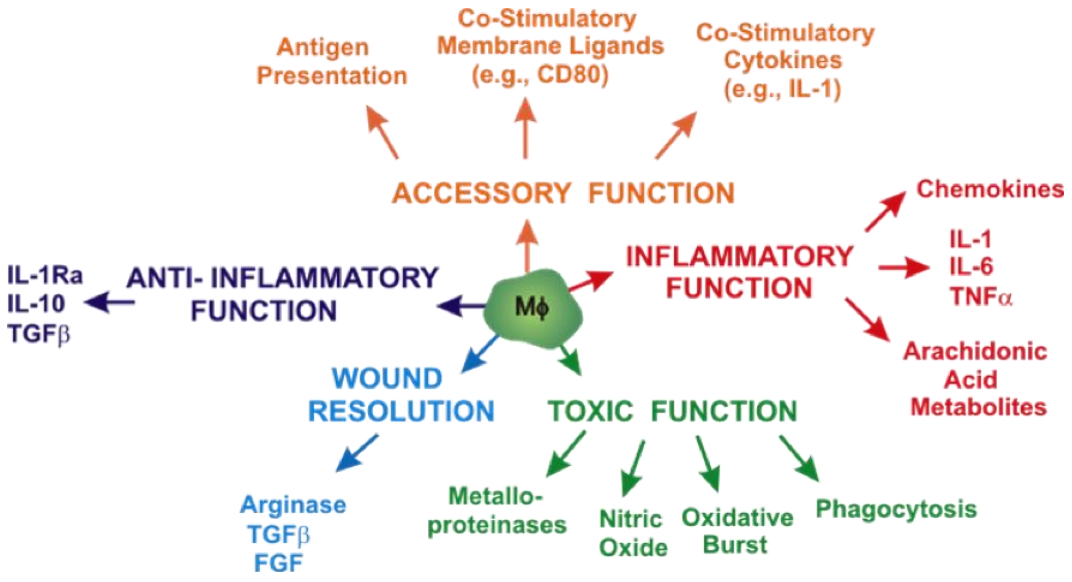

Figure 1. Macrophages perform functional heterogeneity in microenvironment. Macrophages express a variety of functions in response to different changes in their microenvironment. Macrophages can exhibit both proinflammatory and antiinflammatory activities such as secretion of proinflammatory cytokines, chemokine, metabolites, and antiinflammatory cytokines including IL-10 and TGFβ.

acute or chronic inflammation, antigen presentation, wound healing, cytotoxicity, *etc*. (Fig. 1).

Macrophage activation and polarization

Macrophage activation was originally categorized in two distinct states of polarization: the classically activated macrophage phenotype and the alternatively activated macrophage phenotype. Before the publication in 1992 of Stein *et al*., demonstrating a newly discovered effect that IL-4 stimulation enhanced mannose receptor expression in macrophages (4), research was focused on macrophage activation by Th1 cytokine IFNγ and TNFα to enhance macrophage cytotoxicity (24). This discovery, along with subsequent studies, established that IL-4 stimulation is able to up-regulate a distinct set of genes in macrophages. These IL-4 activated macrophages were then defined as "alternatively activated" macrophages, in contrast to the previously described IFNγ/TNFα activated macrophages, referred to as "classically activated" macrophages (25). Based on the cytokines that are associated with these macrophage phenotypes, the nomenclature of M1/M2 was designated to the classically or alternatively activated macrophages, respectively, mirroring the T helper type 1 (Th1)/T helper type 2 (Th2) paradigm. The classically activated macrophages (M1) are usually modeled by *in vitro* stimulation with IFNγ/or LPS stimulation, and are associated with increased microbicidal activity, antigen-presenting functions, and tissue destructive functions. They express elevated genes that are associated with increased proinflammatory features such as MHC class II molecules, nitric oxide synthase 2 (NOS2), IL-12, TNFα, IL-6, *etc*. The alternatively activated macrophages (M2) are usually modeled by *in vitro* stimulation

with IL-4 or IL-13, and are associated with the antiinflammatory functions linked to wound healing, fibrosis, tissue repair, allergy, parasite infection, and tumor progression. Elevated antiinflammatory gene expression was observed in M2 including IL-10, TGFβ, arginase 1 (Arg 1), and mannose receptor 1 (MR), *etc*. (26, 27).

Although the M1/M2 classification persists, a growing body of research suggests much more complex macrophage activation/polarization paradigms. For example, a shift of inflammatory M1 phenotype to antiinflammatory M2 phenotype of macrophages was observed in many disease courses such as sepsis, cancer, obesity, and bacteria infection (26). Combination of different stimuli, such as immune complexes + LPS/IL-1, IL-10 + LPS, IL-10 + IL-4 and IL-13, induced macrophage expression of subset of genes that are shared by both M1 and M2 phenotype (28). Vastly different gene expression was found in macrophages isolated from different mouse strains, and in macrophages stimulated with LPS for short (0-6h) versus long (12-24h) time span (29), suggesting that the functional changes expressed by macrophages in response to stimuli is a response process rather than static. This is also evidenced by the report that the production of IL-12 and TNFα induced by LPS stimulation is significantly repressed by adding IL-4 simultaneously into the macrophage culture, whereas pre-treatment with IL-4 for > 20 h prior to LPS stimulation led to an opposite effect of enhanced IL-12 and TNFα production (30).

Macrophage functional plasticity and regulation

Studies in our lab have demonstrated that macrophages express remarkable heterogeneity and plasticity that is not restricted to the M1/M2 polarization paradigm.

This enormous variety of functional patterns expressed by macrophages is highly dependent on the microenvironment. In this context, different organs and tissues are viewed as distinct microenvironments. For example, the burden of endotoxin and particulate material from the portal circulation in liver provides a highly proinflammatory microenvironment, which is significantly different than the antiinflammatory microenvironment presented by immune privileged sites such as the eye and the brain. These distinct microenvironments provide unique subsets of stimuli that favor macrophage expression of organ specific functions (1). This perspective is supported by our observation of macrophage phenotypic shifts with changes in the microenvironment (7). By adding a variety of stimuli including IFNγ, IL-4, IL-10, IL-12, and FcR ligand for different durations pre- or post- LPS stimulation, macrophages express different cytokine expression patterns that cannot be classified entirely as M1 or M2. These functional patterns of macrophages could be switched by alterations in the microenvironment by adding different combination of the stimulating reagents. And these phenotypic changes could be reverted back to the original, untreated state by removing the reagent treatment from the culture for 1-2 days. These data clearly demonstrated a remarkable ability of macrophages to reversibly adapt to the microenvironment (7), and raised the question of the macrophage heterogeneity over the whole body is a result of differentiation of distinct subsets or a result of differential regulation by the microenvironment.

With the goal of identifying the mechanisms that manage macrophage functional plasticity, we found a master regulator of macrophage functions, AMP-activated protein kinase (AMPK) (14). We found that pro-inflammatory stimuli such as LPS and TNFα

quickly deactivated AMPK while stimulation with the antiinflammatory stimuli such as IL-10, TGFβ, and IL-4 increased AMPK activation. Activation of AMPK leads to inhibition of LPS-induced proinflammatory signaling pathways and cytokine production. Elevated AMPK activity promoted production of antiinflammatory cytokine production in macrophages, polarizing macrophages towards an antiinflammatory direction (14). This study has continued our keen interest in seeking the role of AMPK in regulating macrophage inflammatory plasticity in homestasis and in disease.

AMPK

AMPK is a heterotrimeric protein kinase complex. Its orthologous are expressed with highly conserved features in almost all eukaryotes, mammals, fungi, and even in plants (31). This indicates that its ancestral form emerged at least a billion years ago. The discovery of AMPK goes back to 1970s, when microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase) (32) and acetyl-CoA carboxylase (ACC) (33) were found to be regulated by an upstream regulatory kinase by phosphorylation/ inactivation respectively, both of which were stimulated by AMP. Later in 1987, Carling et al., provided evidence to show that both HMG-CoA reductase and ACC were regulated by the same protein kinase, namely AMP-activated protein kinase (AMPK) (34), and this name became official (35, 36). The structural analysis of mammalian AMPK was achieved based on the successful purification and sequencing of this protein kinase in 1994 (37, 38). Since the identification of AMPK, a burst of research emerged in the past decades exploring its regulation and function. Based on its remarkable ability to sense reduced cellular energy represented by a low AMP/ATP ratio

(39), resulting in shutting off ATP-consuming biosynthetic pathways while turning on ATP-generating catabolic pathways (31, 40, 41), most of the research has focused on the role of AMPK as an energy gauge to regulate metabolic biological processes in the body. Interestingly, recent studies also suggest an important role of AMPK in regulating inflammatory responses and diseases including atherosclerosis, obesity, cancer, *etc*. (10, 11).

AMPK structure and expression

The AMPK heterotrimeric complex contains a catalytic subunit (α) and two regulatory subunits (β and γ). Each subunit has different isoforms in mammalian cells encoded by distinct genes that are distributed across five chromosomes. The corresponding genes that are related to the subunit isoforms (α1, α2, β1, β2, γ1, γ2, γ3) are named PRKA following by the subunit identifier (PRKAA1, PRKAA2, PRKAB1, PRKAB2, PRKAG1, PRKAG2, PRKAG3) (42).

These subunits are differentially expressed in different tissues. The AMPKα1 isoform is evenly distributed across liver, kidney, lung, heart, red vastus, and brain, with higher levels in adipose tissue and lower levels in white vastus, spleen, and pancreas. The AMPKα2 isoform shows highest expression levels in the red vastus, followed by white vastus then heart kidney and liver, with very low levels seen in the lung, brain, and adipose tissue and negligible amounts in the pancreas and spleen (42). We reported in 2008 that the AMPKα1 isoform is highly expressed in both human and murine macrophages with no detectable AMPKα2 isoform expression (14). The AMPKβ1 subunit is widely expressed allover the body and AMPKβ2 subunit has high expression

profile in skeletal muscle and heart to a lesser extent. The AMPKγ1 subunit is ubiquitously expressed. The AMPKγ2 isoform is also widely expressed but highest in the heart, followed by brain, placenta, and skeletal muscle. The AMPKγ3 isoform shows the most restricted expression, as it is confined to skeletal muscle (42).

The mammalian AMPKαβγ heterotrimer exists in solution as a single monomer. Structural analysis revealed the α subunit contains an NH2-terminal kinase catalytic domain, an auto-inhibitory sequence, and a β subunit-binding domain. The β subunit mainly serves as a subunit-binding platform that contains both α and γ subunits-binding domains. The γ subunit contains four adenine-nucleotide binding sites (CBS domains). Other than one out the four motifs (CBS2) that doesn't bind to nucleotide, two of them (CBS1 and CBS3) allow allosteric AMP binding and ready ATP exchange and one (CBS4) allows only nonexchangeable AMP binding with approximately 30-fold stronger binding affinity (41-43). Recent studies revealed that binding of AMP to CBS1 mediates allosteric activation of AMPK, whereas binding of AMP or ADP to CBS3 is correlated with ADP protective effect against dephosphorylation (43-45).

AMPK activation and regulation

It is well established that binding of AMP or closely related AMP analogues to the AMPKγ subunit induces allosteric activation of AMPK by up to ~5 fold (10, 45). In addition to its allosteric activation effect, binding of AMP was also proposed to promote the phosphorylation of the AMPKα1 Thr172 residue (46), although the underlying mechanism was unclear until a recent report in 2011 by Xiao *et al.*, suggesting individual roles of AMP or ADP binding to CBS1 or CBS3 in AMPKγ subunit (44). This study

provided evidence that a tighter association of AMP to the CBS1 binding site is responsible for AMPK allosteric activation, while AMP or ADP binding to the relatively loose binding site CBS3 induces a conformational change of AMPK that promotes phosphorylation on AMPKα1 Thr172 residue and protects against dephosphorylation.

Compared to allosteric activation, AMPK activation by upstream kinase phosphorylation at the Thr172 residue within the activation loop segment of α1 subunit catalytic domain reaches a much higher degree of more than 1000 fold (47). AMPK that is not phosphorylated at α1 Thr172 displays negligible kinase activity (48). Therefore, this phosphorylation/dephosphorylation mechanism plays a major role in regulating AMPK activation/inactivation.

In mammalian cells the three predominant upstream kinases responsible for phosphorylation of Thr172 are liver kinase B1 (LKB1), Ca^{2+} /calmodulin-dependent protein kinase kinase β (CaMKKβ), and transforming growth factor (TGF)-β-activated kinase-1 (Tak1 kinase) (42). LKB1 is constitutively active in cells, and the molecular mechanism of LKB1 phosphorylation of AMPK is unclear (49). The role of LKB1 in activating AMPK has been demonstrated via different methods. LKB1 activation of AMPK *in vitro* was demonstrated by immunoprecipitation and kinase activity assays, and pharmaceutical activators failed to induce AMPK kinase activity in LKB1-deficient murine embryonic fibroblasts (MEFs) (50). Several AMPK-related kinase activities are stimulated by LKB1 (49), and blockage of LKB1 activity in cells abolishes AMPK activation in response to different stimuli (51). In addition to LKB1, CaMKKβ is considered another important AMPK upstream kinase in many cell types in different tissues. CaMKKβ is activated by calcium and calmodulin (52), and treatment with

ionomycin, which increases intracellular Ca^{2+}, led to increased phosphorylation of AMPKα Thr172, a response inhibited by the treatment with the CaMKKβ inhibitor STO-609 in HeLa and A549 cells (53). Increased AMPK activity was observed in mammalian cells with overexpression of CaMKKβ (54), whereas treatment with CaMKKβ siRNA or pharmaceutical inhibitors abolished AMPK activation (54, 55). Other studies supporting CaMKKβ-dependent AMPK activation in response to different agents were also reported in cell types including endothelial cells (56), T cells (57), and neurons (58). Tak1 kinase belongs to the mitogen-activated protein 3-kinase family and was recently suggested to regulate AMPK activities (59). Recombinant Tak1, fused to the activation domain of its binding partner TAB1, was able to induce AMPKα Thr172 phosphorylation *in vitro* and elevated Thr172 phosphorylation was detected in HeLa cells transfected with plasmids to transiently express Tak1 or TAB1 (59). Loss of Tak1 expression in MEFs blocked AMPKα Thr172 phosphorylation and activity induced by the three pharmaceutical activators tested (oligomycin, metformin, and AICAR) (60). However, the ability of Tak1 kinase to act an upstream kinase to regulate AMPK activation was challenged by a study by Kim *et al*. (61). In this study a direct interaction between Tak1 and AMPK was observed in THP-1 cells by co-immunoprecipitation, and both LPS-induced Tak1-Thr187 phosphorylation and Tak1 activity were reduced in AMPKα1 siRNA-containing lentiviruses transfected THP-1 cells, proposing an upstream role of AMPK to regulate Tak1 activity (61). The role of Tak1 as an upstream kinase of AMPK activation is therefore controversial and requires further investigation.

The identification of phosphatases that dephosphorylate the AMPKα subunit and inactivate AMPK has received less attention. In addition to the understanding that

AMPK/ADP-binding to the AMPKγ CBS3 domain confers protection against AMPK dephosphorylation (44), it seems likely the phosphatases involved in the dephosphorylation of AMPK are stimuli and/or cell type-dependent. *In vitro* studies have identified the ability to dephosphorylate AMPKα-Thr172 of members in the protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), both from PPP (phosphoprotein phosphatase) family (35) and protein phosphatase 2C (PP2C), PPM (metal-dependent protein phosphatase) family (46), while specific inhibition of the PPP family by okadaic acid did not affect AMPK activity in rat hepatocytes (62, 63), suggesting that AMPK dephosphorylation is catalyzed primarily by one or more members of the PPM family. However, inhibition of the PPP family phosphatase activities by siRNA knockdown is also effective to block glucose-induced AMPKα-Thr172 dephosphorylation in murine pancreatic β cell line (64). Therefore, the identification of phosphatases involved in cell-type and/or stimulus-dependent AMPK dephosphorylation effect is ongoing.

A wide range of physiological stresses regulate AMPK activity, for example muscle contraction, glucose deprivation, oxidative stresses, treatment with heat shock proteins, *etc*. (65). A variety of agents are recognized as AMPK activators and become pharmacological treatments for many diseases like type 2 diabetes (T2D), obesity, cancer, and atherosclerosis (10, 66, 67). These activators include 5-aminoimidazole-4-carboxamide riboside (AICAR) (68), a nucleoside converted within the cell to an AMP mimetic, metformin (69) and thiazolidinediones (TZDs, such as rosiglitazone), both drugs used to treat T2D and atherosclerosis (70). Different mechanisms were proposed for the action of those activators including increased AMP and/or ADP levels, activation of AMPK upstream kinase such as CaMKKβ, or other enzymes that may lead to

accumulation of cellular cAMP (10). Understanding the pharmacological mechanisms of these AMPK-activating drugs to treat disease still needs investigation, as both AMPK-dependent (68) and AMPK-independent (71) effects of these drugs were reported.

Notably hormones and cytokines are also reported to regulate AMPK activity (72-75). Adiponectin and leptin are both hormones secreted by adipose tissue that play important roles in regulating energy homeostasis and glucose and lipid metabolism. AMPK can be activated by stimulation with adiponectin and this effect is responsible for adiponectin induced fatty-acid oxidation and glucose uptake in both liver and muscle cells (76). Leptin also induces fatty acid oxidation and glucose uptake in muscle, and leptin activation of AMPK is required for this action (77). In leukocytes, stimulation with adiponectin resulted in increased production of antiinflammatory cytokine IL-10 (78), similar to the effect of AMPK activation in macrophages that we reported previously (14). Cytokines such as TNFα and IL-6 are also reported to modulate AMPK activity. Chronic treatment with TNFα leads to suppression of AMPK activation in muscle cells via transcriptional up-regulation of PP2C (79). Stimulation with IL-6 rapidly increased AMPK activation in myotubes (80), and this enhancement of AMPK activity by IL-6 treatment was also observed in muscle and adipocytes (81).

The published research in our lab revealed a key regulatory role of AMPK in macrophage phenotypic activation in response to physiological stimuli with pro- or anti-inflammatory functions (14). In this research, AMPKα1 was shown to be the major isoform expressed in macrophages. Phosphorylation on AMPKα-Thr172 residue was quickly and robustly increased in macrophages by stimulation with IL-10 or TGFβ, both

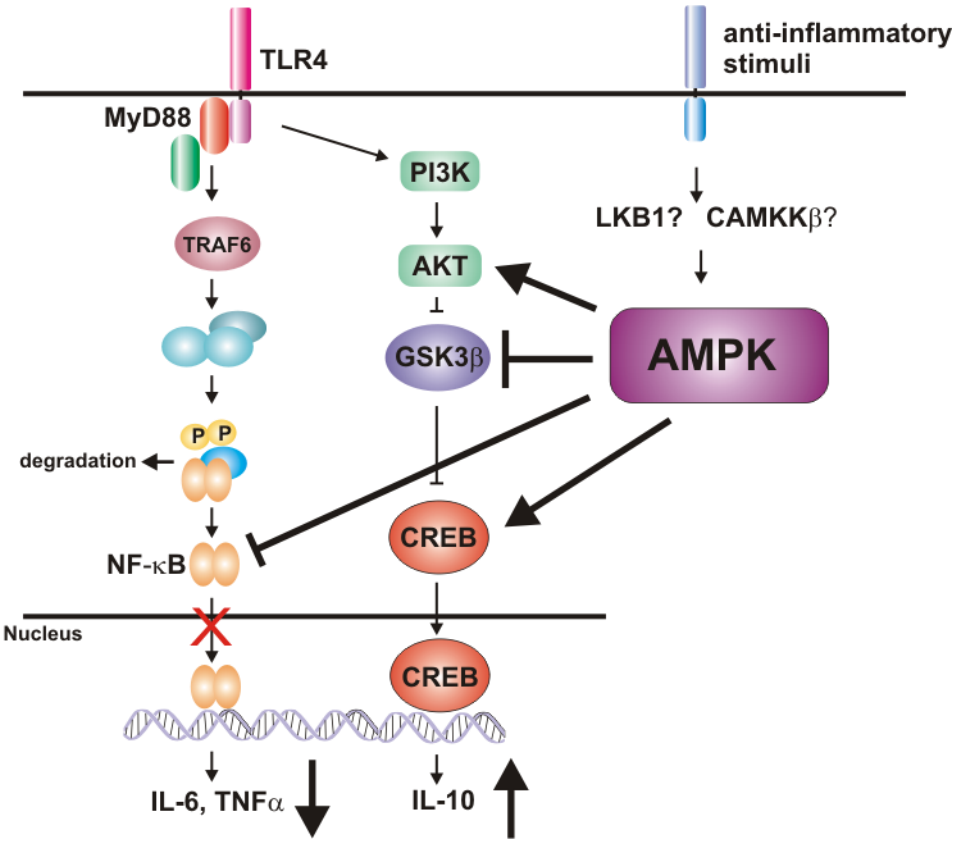

Figure 2. AMPK is activated by antiinflammatory stimuli and suppresses the LPS-induced NF-κB pathway in macrophages. Stimulation with IL-10 activates AMPK. In macrophages stimulated with LPS, AMPK suppresses proinflammatory cytokine production by inhibition of NF-κB pathway, whereas it promotes antiinflammatory cytokine IL-10 production.

antiinflammatory cytokines, whereas stimulation with TNFα or LPS reduced AMPK activation in macrophages. Forced expression of CA-AMPKα1 in macrophages led to increased IL-10 production while decreased proinflammatory cytokine production in response to LPS, whereas forced expression of DN-AMPKα1 led to the opposite effect in macrophages. This ability of activated AMPK to promote macrophage antiinflammatory functions is supported by mechanistic studies, which showed that the LPS-induced NF-κB signaling pathway activation was greatly suppressed in the CA-AMPKα1 macrophages while the opposite occurred in the DN-AMPKα1 macrophages. This study promoted the theory that the pro- or anti- inflammatory physiological stimuli in the microenvironment are able to antagonize or promote AMPK activation, leading to enhanced or suppressed macrophage inflammatory function, respectively (Fig. 2). This previous study provided the theoretical foundation of this Ph.D. dissertation research investigating the underlying mechanism of AMPK regulation of IL-10-induced immune suppressive signaling pathways, which lead to macrophage activation towards an antiinflammatory state.

AMPK in metabolic syndromes

The exclusive role of AMPK in maintaining energy homeostasis over the whole body has implications for its role in metabolic syndromes such as insulin resistance, T2D, cardiovascular disease, and fatty liver disease. To sustain metabolism, the intracellular ATP concentration must be regulated within an appropriate range in the body at both subcellular and systemic levels. As a tissue that constantly requires significant variation in ATP synthesis, skeletal muscle serves as a good target to understand AMPK regulation

of metabolic disorders (82). Several beneficial drugs such as polyphenols (83) and berberine (84) used to treat metabolic disorders stimulate AMPK activity in skeletal muscle. Improved AMPK activity is responsible for PPARγ coactivator-1α (PGC-1α)/ glucose transporter 4 (GLUT-4) activation/expression in muscle cells to promote glucose uptake and fatty acid oxidation (85), effects that contribute to improvements in insulin sensitivity and obesity.

The AMPK-mediated gene regulation related to glucose uptake and lipid oxidation is also seen in liver, an organ that plays pivotal role in the control of whole body metabolism of energy nutrients (86). The first two AMPK downstream substrates identified, ACC and HMG-CoA reductase, are key enzymes in the regulation of fatty acid and cholesterol synthesis, respectively, in the liver. Hepatic AMPK can be activated by physiological stimuli such as exercise and nutrient deprivation, physiopathological situations such as prolonged starvation or chronic alcohol consumption, and pharmacological drugs such as metformin and TZDs (70, 87). Activation of hepatic AMPK leads to inhibition of ACC and enhanced fatty acid oxidation (88), inhibition of HMG-CoA reductase and reduced cholesterol synthesis (89), and inhibition of the TSC2/mTOR pathway and decreased protein synthesis (90). Meanwhile, hepatic AMPK also suppresses the expression of glycolytic and lipogenic genes such as liver-type pyruvate kinase (L-PK), fatty acid synthase (FAS) (91), sterol regulatory element-binding protein-1c (SREBP-1c), and carbohydrate response element-binding protein (ChREBP) (92), as well as gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) (92). Increased hepatic glucose production is an important hallmark of T2D. Suppression of gluconeogenesis, a key

metabolic pathway for glucose output in liver, plays a beneficial role in improving overall glycemic control in T2D. Treatment with AMPK activator abolishes hyperglycemia in diabetic ob/ob mice (92). Hepatic AMPK therefore provides a valuable therapeutic target for the management of metabolic hepatic disorders linked to T2D and obesity.

Adipose tissue is composed of adipocytes, endothelial cells, fibroblasts and macrophages. It plays a key role in energy storage and is well recognized as an endocrine organ secreting the anorexigenic cytokine, leptin, and the insulin-sensitizing hormone, adiponectin (75). During exercise, fasting and in the presence of hypoglycemic drugs, AMPK can be activated in adipose tissue. Leptin and adiponectin are both activators of AMPK (76, 77, 93, 94). As in the liver, activation of AMPK in adipose tissue suppresses ACC activity and the expression of lipogenic genes thus favors local fatty acid oxidation while repressing lipogenic fluxes. In addition, activation of AMPK leads to limitation of lipolysis, a process that the adipose tissue employs to break down the triglycerides through the lipolytic pathway during fasting to provide fatty acids and glycerol as fuels for peripheral tissues (95). Treatment with the AMPK activator AICAR inhibited the secretion of TNFα and IL-6 in adipose tissue (96, 97). To summarize, adipose tissue AMPK tends to enhance local fatty acid oxidation while inhibiting fatty acid and triglyceride synthesis and lipolysis to limit fatty acid efflux from adipocytes to the plasma. This AMPK effect is extremely beneficial in insulin-resistant states such as T2D, particularly as AMPK activation also reduces inflammatory cytokine secretion by adipocytes.

AMPK in inflammation

It is known that metabolic syndromes, particularly obesity, T2D, fatty liver disease and atherosclerosis, are associated with chronic inflammation (98). The ability to withstand starvation and the capacity to mount an effective immune response to pathogens are among the most fundamental requirements for species survival. The two systems, metabolism and inflammation, are highly integrated and the proper function of each is dependent on the other. Evolutionarily, the functional units that control key metabolic and immune functions in higher organisms have evolved from common ancestral structures such as the *Drosophila* fat body, a structure found in lower organism *Drosophila melanogaster* which incorporates the mammalian homologues of the liver and the hematopoietic and immune systems (98, 99). The association between metabolism and inflammation is also evident in metabolic important tissues of higher organisms, for example the liver and adipose tissue where macrophages reside along with hepatocytes and adipocytes. The immune effector cell macrophages and the metabolic important cell adipocytes show evidence of coordination and coevolution. Both of these two cell types share common functions such as cytokine secretion and responsiveness to LPS (100). This coevolutionary relationship is also supported by the evidence that a wide range of overlapping gene expression is found between macrophages and adipocytes, and many adipocyte-specific metabolic and other genes are upregulated during the transformation of macrophages into foam cells (101). Given the critical role of AMPK in metabolic syndromes, it is not surprising the regulation of inflammatory responses also requires AMPK activity.

The beneficial role of AMPK activator AICAR is showed in experimental

autoimmune encephalomyelitis (EAE) (102) and colitis (103), both disease models associated with chronic inflammation. Metformin, an effective anti-diabetic drug and AMPK activator, confers antiinflammatory features in impaired glucose tolerance (IGT) patients (104), IL-1β treated human vascular wall cells (105), LPS or oxidized LDL treated human monocytes (106), and endotoxin-induced uveitis rat model (107). Chronic treatment with TNFα leads to suppression of AMPK activation in muscle cells via transcriptional up-regulation of PP2C, an effect that is associated with downregulated fatty acid oxidation (79), and neutralization of TNFα leads to elevated AMPK activity in a murine obese model. This study provides evidence supporting an AMPK-regulated mechanism to link inflammation and defective fatty acid metabolism in obesity.

Our report in 2008 was the first investigation of AMPK activity in immune cells (14). Macrophages are important players in both innate and adaptive immune responses. The accumulation of macrophages in adipose tissue in an inflammatory state is a hallmark of obesity-induced insulin resistance. Our study showed that AMPK activation is directly triggered by antiinflammatory stimuli such as IL-10, TGFβ, and IL-4, whereas it is suppressed by proinflammatory stimuli such as LPS and TNFα in macrophages. Activated AMPK then acts as a key regulator to promote macrophage antiinflammatory functions via stimulation of the Akt/CREB pathway and inhibition of the IKK/IκB/NF-κB pathway (14). A number of studies then emerged after our initial findings to further demonstrate an important regulatory role of AMPK in macrophages (108, 109), dendritic cells (DCs) (15, 110), and T cells (111-113). Macrophages deficient in AMPKβ1 expression displayed increased levels of diacylglycerol and markers of inflammation, and hematopoietic deletion of AMPK β1 results in systemic inflammation and insulin

resistance in adipose and hepatic tissue in mice fed on high fat diet (HFD) (108), revealing a protective role of myeloid AMPK against inflammation and insulin resistance during obesity. AMPK activity is reduced in macrophages treated with fatty acids and HFD (109). In an adipocyte/macrophage co-culture system, inactivation of AMPKα1 in macrophages resulted in reduced insulin signaling in adipocytes. This research also showed that macrophage AMPKα1 negatively regulates the inflammatory response caused by exposure to free fatty acids, LPS or diet-induced obesity by inhibiting NF-κB signaling in via SIRT1 deacetylation of the NF-κB p65 subunit (109). In addition to the key regulatory role of AMPK in macrophage antiinflammatory functions (14), we also reported a positive role of AMPK to suppress CD40-mediated inflammatory responses in DCs (15). AMPK-deficiency in both macrophages and DCs lead to increased IL-6 and TNFα production, whereas IL-10 production was decreased in response to LPS stimulation. The antigen-induced Th1 and Th17 responses were enhanced in T cells co-cultured with AMPKα1-deficient macrophages and DCs. Upon treatment of CD40 ligand, CD145, stimulation, AMPKα1-deficiency was shown to limit Akt/CREB pathway activation while enhancing NF-κB and MAPK pathways therefore enhancing CD40-induced proinflammatory responses in DCs (15). LPS stimulation was shown to reduce AMPK activation in DCs and this effect was accompanied by increased cellular glucose consumption (110). In addition to our findings that AMPK-deficiency in both antigen presenting cells (APCs) and T cells greatly enhanced Th1 and Th17 responses, AMPK is also shown to improve T cell viability in the absence of survival signals such as IL-2, and to suppress GLUT-1 expression and to limit glycolysis in T cells (111). Significantly, elevated AMPK activity is found in T regulatory cells (Treg) compared to T effector cells

(Teff), and treatment with metformin improves Treg frequency (112). Most recently, AMPKα1 expression was found essential for generation of CD8+ T cell memory (113).

In contrast to the long road discovering AMPK functions in metabolic systems since the 1970s, the understanding of AMPK function in immune responses is relatively nascent, but quickly developing. Research in our lab has been conducted with keen interest in exploring mediators of immune cell functional plasticity. During the discovery of fatty acid binding protein (FABP) regulation of macrophage phenotype the significant role of AMPK was noticed. Investigation of AMPK regulation of immune cell functions including macrophages, DCs and T cells have been developed in our lab and important roles of AMPK in the regulation of immune responses has been revealed. The underlying mechanisms responsible for these AMPK-mediated effects in different immune cells need to be further defined. Herein, we provide evidence to support an important role of macrophage expressed AMPK in the expression of atheroprotective proteins and demonstrate a key role of AMPK to integrate and regulate IL-10-induced signaling pathways that lead to functional reprogramming of macrophage functions towards an antiinflammatory direction. These studies underscore the value of AMPK as a therapeutic target for chronic inflammation-associated diseases such as atherosclerosis, autoimmune disease and cancer.

CHAPTER 2

AMPK REGULATION OF MACROPHAGE FUNCTION IN ATHEROSCLEROSIS

INTRODUCTION

Atherosclerosis is commonly associated with metabolic syndromes. Statistics in 2010 implicate that atherosclerosis is the leading cause of death and morbidity in developed countries and is likely soon to attain this status worldwide (114). Atherosclerosis is characterized by a lipid metabolic imbalance that results in accumulation of lipids and fibrous elements at the artery wall. This process leads to a chronic inflammatory process and to the development of complex lesions, or plaques, that protrude into the arterial lumen and induce acute clinical complications of myocardial infarction and stroke (115). In the body, dietary and endogenous cholesterol is carried and transported by several lipoprotein particles including chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The majority of serum cholesterol is carried by LDL, which can be transformed from VLDL by removal of the triglyceride content from the particle. LDL is subject to oxidative modifications in the subendothelial space, and progresses from minimally modified LDL (mmLDL) to extensively oxidized LDL (oxLDL) (116). Cholesterol

within LDL droplets can be internalized by the peripheral cells expressing LDL receptor (LDLR) via receptor recognition, whereas modified/oxidized LDL particles can only be recognized by a group of scavenger receptors such as SR-A and CD36. While high LDL cholesterol level is associated with high risk of cardiovascular disease, lack of LDLR expression in mice could significantly exacerbate plasma cholesterol level induced by HFD and result in massive hypercholesterolemia leading to extensive atherosclerotic disease (117). Accumulation of modified LDL in the artery wall results in endothelial cell expression of adhesion molecules like VCAM-1 and monocyte-attractive chemokine like MCP-1. Circulating monocytes are attracted to the artery wall directly by ox-LDL and by endothelial-secreted MCP-1 and attach to the endothelial layer via the adhesion molecules. The adherent monocytes then migrate into the subendothelial space and differentiate into macrophages. At the intima, macrophages take up ox-LDL via scavenger receptors and transform to foam cells. This process is so called "fatty streak lesion" formation, and initiates the first steps of atherosclerosis. The development of lesion progression is mediated by a chronic state of inflammation created by interactions between macrophage-derived foam cells and T cells. Cytokines secreted by lymphocytes and macrophages exert both pro- and anti- atherogenic effects on each of the cellular elements of the vessel wall. Smooth muscle cells (SMCs) migrate from the medial portion of the arterial wall to the lesion where they proliferate and secrete extracellular matrix (ECM) proteins that form a fibrous plaque. With growing burden of the lesion, macrophage- and SMC-derived foam cells undergo necrotic death and release the lipid-rich cell contents to the necrotic core of the lesion. The lesions continue to grow by the recruitment of new monocytes to the lesion, accompanied by cell proliferation, ECM

protein production and the accumulation of extracellular lipid. During this process, macrophage secretion of matrix metalloproteinases (MMPs) and neovascularization contribute to weakening of the fibrous plaque. Eventually, the plaque ruptures and exposes blood components to tissue factor, which initiates coagulation, the recruitment of platelets, and the formation of a thrombus, resulting in myocardial infarction or stroke (115, 118).

MACROPHAGES AND AMPK IN ATHEROSCLEROSIS

Macrophages in atherosclerosis

Atherosclerosis was once considered to be a cholesterol storage disease characterized by the collection of cholesterol and thrombotic debris in the artery wall. With the identification of abundant monocyte-derived macrophages in atherosclerotic plaques, and the gradual recognition of their importance in atherogenesis, macrophage-mediated innate and adaptive immunity provided modern insights to the research of atherosclerosis as a complex chronic inflammatory disease (118-122).

Macrophages play important roles in all steps of atherosclerosis progression, from lesion formation to plaque destabilization. A hallmark of atherosclerosis is the transformation from monocyte-derived macrophages to lipid-laden foam cells in the subendothelial intima. Although macrophage take-up of lipoproteins is initially beneficial, lack of negative feedback mechanisms results in accumulation of lipids within those macrophages. This leads to dysregulation of lipid metabolism and alters the immune features of macrophages, both in proliferative functions and inflammatory phenotypes, therefore promoting the development of the plaque (17, 123, 124). These

macrophages continue to establish a chronic inflammatory response with the participation of other local or recruited immune cells to the lesion by secreting proinflammatory mediators including chemokine, cytokines, reactive oxygen and nitrogen species, and matrix-degrading proteases. Eventually the death of macrophages causes the release of the tissue factor- and lipid-rich contents which leads to the formation of necrotic core, a key component of unstable plaques. The rupture of the unstable plaque is the final cause of myocardial infarction and stroke (17, 125).

Although many immune cell types exhibit heterogeneity during atherosclerosis such as monocytes and DCs, the central role of macrophage heterogeneity and functional phenotypic change has been highlighted in a growing body of evidence (17, 125, 126). A broad range of macrophage heterogeneity is found in atherosclerotic lesions at different plaque development stages (127), with proinflammatory macrophage phenotypes found to be enriched in progressing plaques and antiinflammatory macrophage phenotypes found to be enriched in regressing plaques. Moreover, phenotypic changes from an antiatherogenic "M2-like" state of activation to a proatherogenic "M1-like" state of activation is observed during atherosclerosis progression (128). We previously described a remarkable macrophage plasticity to be differentially activated by microenvironmental factors (1, 7, 8). The diverse plaque-associated environmental signals, even with opposing immune features, may play critical role in directing macrophage polarization during lesion development. Indeed, a high level of IL-4 in early stage lesions, and a high level of IFNγ in late stage lesions, is found to associate with the phenotypic switch from "M2-like" activation state to "M1-like" activation state of macrophages during atherosclerosis progresssion (127), and oxLDL is reported to modulate macrophage

activation as well as induce unique gene expression patterns (129, 130). These studies suggest the environmental factors during atherosclerosis lesion progression contribute siginificantly to macrophage activation and phenotypic polarization, therefore modulating plaque development.

Given the significance of macrophages, many regulators of macrophage cholesterol trafficking and macrophage functional polarization have been identified as therapeutic targets to treat atherosclerosis. These targets include growth/differentiation factors such as macrophage colony-stimulating factor M-CSF (125), cytokines such as IL-10 (131-133), lipid chaperones such as fatty acid binding proteins (FABPs) (134), transcription factors such as peroxisome proliferator activated receptors (PPARs) and liver X recepters (LXRs) and subsequent genes including apolipoprotein E (apoE) and ATP-binding cassette transporter A1 (ABCA1) (135). Most recently AMPK is also suggested to play atheroprotective roles (136), although these studies have focused on endothelial inflammation and the endogenous AMPK activation in macrophages received less attention in atherosclerosis research.

Fatty acid-binding proteins (FABPs) in atherosclerosis

FABPs comprise a family of homologous cytosolic proteins with distinct patterns of tissue expression. The capability of FABPs to bind hydrophobic compounds and shuttle them between cytoplasmic compartments and enzyme systems within the cell has implicated FABPs as upstream modulators of many lipid signaling cascades (137-140). Studies on FABP-deficient mouse models reveal a strikingly protective role of FABP-deficiency from development of obesity, insulin resistance, T2D, fatty liver disease and

atherosclerosis (134, 141). A-FABP, also known as aP2 and Adipocyte Lipid Binding Protein (ALBP), is expressed by adipose tissue, adipogenic cell lines, and macrophages. A-FABP-deficiency provides remarkable protection against atherosclerosis in apoE-deficient models of atherosclerosis, and this effect of A-FABP is predominantly related to its actions in the macrophage (142, 143). Likewise, treatment with A-FABP inhibitors specifically targeting macrophages protects mice against severe atherosclerosis (144). The collaborative work between Suttles' lab and Hotamisligil's lab provided the underlying mechanism of A-FABP to control macrophage cholesterol trafficking and inflammatory activity via impairment of PPARγ activity that leads to inhibition of CD36 and ABCA1-mediated pathways and enhancement of IKK/NF-κB pathway, respectively (145).

Notably, A-FABP binds to a variety of metabolites including compounds that act as ligands for PPARγ (146, 147). Although there are reports of FABPs shuttling PPAR ligands to the nucleus in non-macrophage cell types (148-150), our previous observation of elevated PPARγ activity in A-FABP-deficient macrophages suggests that in macrophages, FABPs act to sequester PPARγ ligands in the cytosol, thus preventing their migration to the nucleus. When A-FABP expression is absent, lipid ligands are free to interact with nuclear receptors, as well as other targets, including IKK (145). This speculation is supported by the Suttles lab's unpublished confocal microscopy data showing that a PPARγ ligand, 13-HODE, is sequestered in the cytosol of wild-type macrophages whereas is present in the nucleus of A-FABP-deficient macrophages (Fig. 3).

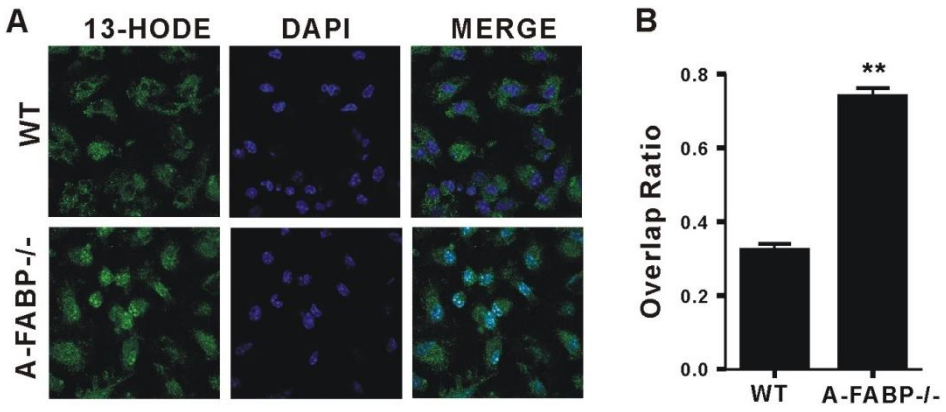

Figure 3. Presence of FABPs prevents nuclear localization of 13-HODE. (**A**), 13-HODE (10μM) (green) was restricted to the cytoplasm of macrophages in the presence of A-FABP expression, but was able to enter the nucleus in A-FABP-deficient macrophages (turquoise). (**B**), Quantitation of co-localization of 13-HODE in nucleus (**p<0.001).

Peroxisome proliferator activated receptor γ (PPARγ) in atherosclerosis

PPARγ is expressed in macrophages and belongs to the PPAR family of nuclear receptors, which are ligand-activated transcription factors that function as fatty acid sensors to regulate glucose and lipid metabolism (151, 152). Studies *in vitro* demonstrated the role of PPARγ to promote cholesterol trafficking in macrophages via transcriptional induction of genes encoding proteins involved in cholesterol trafficking including the scavenger receptor CD36 (153), LXRα and ABCA1 (154). Treatment with PPAR agonists *in vivo* exhibit beneficial effects in atherosclerosis animal models (135). Expression of PPARγ can be induced by IL-4 stimulation in macrophages (155), and activation of PPARγ promotes macrophage activation towards an antiinflammatory state associated with elevated expression of Arg 1, MR, and the antiinflammatory cytokine IL-10 (156-158) while it suppresses activation of proinflammatory pathways and gene expression (155, 159).

Interleukin 10 (IL-10) in atherosclerosis

IL-10 is a potent immunosuppressive cytokine produced by Th2-type T cells, B cells, monocytes, and macrophages that systemically limits excessive inflammation and controls inflammatory disease progression including atherosclerosis (160, 161). It is produced in atherosclerotic lesions and correlates with diminished expression of inflammatory mediators within the lesion and vessel wall (162-164). IL-10-deficient C57BL/6 mice fed on an atherogenic cholate-containing diet develop early atherosclerotic lesions, characterized by increased lipid accumulation, increased infiltration of inflammatory cells, and increased proinflammatory cytokine production

(131, 165). The similar atheroprotective property of IL-10 is demonstrated with IL-10-/-apoE-/- mice fed a chow diet (166). Consistent with this protective role of IL-10 in atherosclerosis, overexpression of IL-10 using the human IL-2 promoter or by adenoviral gene transfection resulted in effective prevention of atherosclerosis development (165, 167). Leukocyte-derived IL-10 is crucial in the prevention of atherosclerotic lesion development as it influences leukocyte functions as well as modulates systemic immune response thus influences plaque composition (168). Most recently, a series of studies by Han et al., established a critical role of IL-10 in promoting cholesterol trafficking in macrophages treated with modified LDL by promoting PPARγ-mediated atheroprotective gene productions whereas inhibiting proinflammatory gene expression (132), and macrophage specific overexpression of IL-10 in mice led to reduced atherosclerosis development (133), suggesting an important role of IL-10-regulated macrophage function in regulating atherosclerosis progression. All the studies analyzing the role of IL-10 in murine atherosclerosis models demonstrated that the regulation of balance between proinflammatory and antiinflammatory cytokines is decisive for disease development and progression (161). Therefore, research seeking for the regulators of this balance, especially in macrophages, is extremely promising as a source of new therapeutic targets to treat atherosclerosis.

AMPK in atherosclerosis

Our research has established a key regulatory role of AMPK in modulating macrophage polarization in response to pro- or anti- inflammatory stimuli that subsequently leads to modulation of the balance between macrophage produced

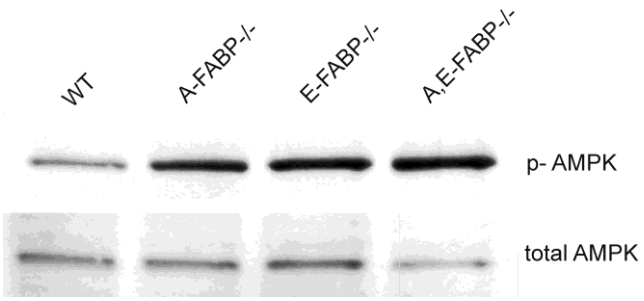

Figure 4. FABP-deficient macrophages express elevated AMPK activity. Macrophages with deficient expression of A-FABP, E-FABP, or both A- and E-FABP show significantly increased AMPK activation compared to wild type macrophages.

proinflammatory and antiinflammatory cytokines (14). Therefore, the AMPK expression in macrophages may play important roles in atherosclerosis. In support of this hypothesis, the atheroprotective role of AMPK has been suggested in recent studies based on the significant role of AMPK in regulating both metabolic fatty acid oxidation pathways and inflammatory pathways (18, 19, 66, 136). Many atheroprotective agents are proved to be potent AMPK activators such as AICAR, metformin, TZDs, statins, and adiponectin. However, the protective role of AMPK has not been investigated until recent, and most of the studies concerning this topic focus on the AMPK regulation of metabolic processes in non-immune cells such as in endothelial cells and SMCs. The underlying mechanism of this AMPK atheroprotective effect is not well demonstrated and the role of macrophage expressed-AMPK in the regulation of atherosclerosis progression has not been reported so far.

During the investigation of FABP regulation of macrophage functions (145), we found a striking effect that FABP-deficient macrophages display greatly enhanced PPARγ activity accompanied by elevated IL-10 production, and significantly enhanced AMPK activity (Fig. 4). We have also shown that FABPs can antagonize PPARγ activity by holding its ligands within the cytosol (Fig. 3). PPARγ is reported to promote antiinflammatory responses in macrophages including the induction of IL-10 expression (158). IL-10 activates AMPK in macrophages in a time-dependent manner (14), AMPK then act as a counter regulator of macrophage inflammatory responses. Interestingly, AMPK has also been implicated in the activation of PPARs, in part due to upregulation of PGC-1 (169). Based on these findings, herein in this study we have demonstrated that

macrophage-expressed AMPK plays an important role in cardiovascular disease and that the systemic protective effect of AMPK activators includes their influence on macrophage AMPK activity. We previously observed greatly increased macrophage expression of FABP in mice feed on HFD; here we provide evidence to support a FABP/ PPARγ pathway that leads to increased IL-10 production resulting in increased AMPK activation, which in turn, promotes PPARγ activity and IL-10 signaling in macrophages and mantains macrophage polarization towards an antiinflammatory, antiatherogenic state.

MATERIAL AND METHODS

Mice

Macrophage-specific constitutively active (CA)-AMPKα1 and dominant negative (DN)-AMPKα1 transgenic mice were provided by Dr. DeBroski Herbert, Division of Immunology, University of Cincinnati College of Medicine. These mice were crossbred with LDLR-/- mice to generate macrophage-specific CA/DN-AMPKα1/LDLR-/- mice. C56BL/6J mice and LDLR-/- were purchased from The Jackson Laboratories. AMPKα1 deficient (AMPKα1-/-) mice were generated as previously described (170). C57BL/6J mice were purchased from the Jackson Laboratory. These mice were bred and maintained in the Research Resources Facility, University of Louisville. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee, University of Louisville.

High Fat Diet procedure

High fat diet (60% kcal from fat, product number D12492) food was purchased from Research Diets, Inc. After being weaned, macrophage-specific CA-AMPKα1 and DN-AMPKα1 transgenic mice with C56BL/6J control mice were placed on the diet for 80 days (10 mice per group). Whole body weight of individual mouse was weighted every two days. Fat tissue was isolated from different parts of the body and weighted before fat associated macrophage (FAM) isolation.

Fat associated macrophage (FAM) isolation

Fat pads were isolated from animal and weighted before fat associated macrophage (FAM) isolation. The fat tissue was then placed into 10 cm petri dish with PBS + 2% FBS. Fatty tissue was cut into small pieces and collected into a 50ml tube and PBS + 2% FBS was added to get a final volume of 30 ml. Samples were centrifuged at 500 g, 4°C for 5 minutes to remove the red blood cells. The floating tissue was transferred to a new tube and collagenase was added for 30-60 minutes at 37°C, depending on the tissue volume. The tube was lightly vortexed every 5-10 minutes for contact. The contents were filtered through a 100um fliter into a 50mL conical tube. Samples were washed 3 times with PBS + 2% FBS by centrifugation at 500g, 4°C for 5 min between washes. After the final wash, the floating tissue and buffer was removed and the SVT pellet was resuspended in fresh buffer. Single-cell suspension was achieved by gentle pipetting. Gey's solution was added for 5 min to remove red blood cells. Samples were then washed 3 times with PBS + 2% FBS by centrifugation at 500g, 4°C for 5 min between washes. Cells were counted for FAM isolation. FAM were isolated by positive selection with anti-CD11b (Mac-1) magnetic beads (Miltenyi Biotec). A purity of $> 95\%$ $CD11b^+$ cells was confirmed by flow cytometry.

Reagents

LPS (Escherichia coli serotype O111:B4) was purchased from Sigma-Aldrich. Recombinant mouse IL-10 was purchased from R&D Systems. Purified NA/LE Rat Anti-Mouse IL-10 neutralization Ab. was purchased from BD Pharmingen™. 15-deoxy-Δ12, 14-PGJ2 (15dPGJ2), 13R-hydroxy-9Z, 11E-octadecadienoic acid (13-HODE),

Rosiglitazone, and GW9662 were purchased from Cayman Chemical (Ann Arbor, MI). Western blot detection of specific proteins used the following primary Abs: anti-A-FABP, anti-E-FABP (R&D Systems), anti-phospho-AMPKα (Thr172), anti-AMPKα (Cell Signaling), anti-β-actin (Sigma-Aldrich), and HRP-conjugated secondary Ab (Jackson ImmunoResearch Laboratories).

Cells

Bone marrow-derived macrophages (BMDM) were generated from C57BL/6J mice following the protocol previously described (171). CA-AMPKα1 and DN-AMPKα1 macrophage stable transfected cell lines were generated following the protocol previously described (14). Cell culture were maintained in incubators set to 37°C and 5% CO_2. For stimuli/reagents used in individual experiments, cells were harvested into a 24-well cell culture plate (1 x 10^6 cells/well) and rested overnight in RPMI medium supplemented with 5% FBS, 10ml HEPES Buffer (Sigma), and 1ml gentamycin (Atlanta Biologicals) (referred as R5 medium) prior to stimulus. The cells were quickly rinsed with pre-warmed DPBS supplemented with 2% FBS before lyse. Kupffer cells were isolated from mouse liver by preparing single cell suspension according to gentle MACS protocol followed by positive selection with anti-CD11b (Mac-1) magnetic beads (Miltenyi Biotec).

RNA interference

Bone marrow-derived macrophages were transfected with 0.5 μg AMPKα1 or PPARγ small interfering RNA (siRNA) or non-targeting control siRNA (Dharmacon, Thermo

Scientific) using Nucleofector™ Kits according to the corresponding Amaxa™ Optimized Protocols (Lonza). Following Nucleofection™, the macrophages were plated in 12-well plates in RPMI 1640 (HyClone) medium containing 20% FBS (Atlanta Biologicals), 10 mM HEPES, and 10 μg/ml gentamicin. The cells were analyzed 24 h or 48 h post-transfection.

Real-time RT-PCR analysis

mMACs™ One-step cDNA Kit (Miltenyi Biotech) were used for RNA isolation and cDNA synthesis. cDNAs were amplified in a 20 μl reaction volume containing SYBR Green (New England Biolabs) and analyzed using a DNA Opticon 2 Monitor (MJ Research). All the gene mRNA expression levels (*abca1*, *apoe*, *socs3*, *socs1*, *nr1h3*, *arg1*, *il-10*, *tgfb*, *tnfa*, *il-6*, and *il-12b*) were analyzed by Quantitect Primer Assays (Qiagen). The cDNA concentrations in each sample were normalized using transcripts for β-actin. The relative expression software tool (REST©) was used to quantify mRNA expression (172).

Western blot analysis

Whole cell lysates were generated by lysis with a buffer containing 125 mM Tris (pH 6.8), 2% SDS, 20% glycerol, 100 μM PMSF, protease inhibitor mixture (Promega), and HALT™ phosphatase inhibitor cocktail (Thermo Scientific). Total protein content of the samples was assessed by BCA protein assay (Pierce). Equal amounts of protein were separated on 10% Criterion gels (Bio-Rad) by SDS-PAGE. Medium and high molecular weight proteins (55-289KDa) were transferred to nitrocellulose membranes using a

Trans-Blot® Turbo™ Nitrocellulose Transfer Pack and Trans-Blot®Turbo™ transfer system (Bio-Rad). Low molecular weight proteins (< 55kDa) were transferred to nitrocellulose membranes (Hybond; Amersham Biosciences) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Ab-bound proteins were detected using an ECL Western blotting analysis system (Amersham Corp.), and the membranes were exposed to SRX-101A film (Konica Minolta). Densitometry analysis was performed using the UN-SCAN-IT gel (version 6.1) software.

ELISA

Following stimulation in 24-well plates, supernatants were collected into 96-well plates and assayed by ELISA using OptEIA™ sets (BD Biosciences Pharmingen) according to the manufacturer's instructions. Analysis was performed using an E-max precision micro plate reader (Molecular Devices).

Statistical Analysis

Statistical significance between groups was calculated with an unpaired Student's *t* test, with a p value < 0.050 considered statistically significant.

RESULTS

Generation of macrophage-specific CA-AMPKα1 and DN-AMPKα1 mice

Our lab previously constructed stable transfected macrophage cell lines with expression of either CA-AMPKα1 or DN-AMPKα1 by nucleotransfection (14). The CA-AMPKα1 containing constructs encode an α1 subunit with a polypeptide truncated at residue 312 so it no longer associates with the β and γ subunits but retains significant kinase activity. The DN-AMPKα1 containing constructs encode an α1 subunit with mutation of aspartate 157 to alanine in a conserved subdomain that yields an inactive kinase but does not have any effect on the binding of the β and γ subunits within the complex (173). To generate macrophage-specific CA-AMPKα1 and DN-AMPKα1 transgenic mice for the purpose of this study, these constructs were sent to Dr. DeBroski Herbert, University of Cincinnati College of Medicine. Dr. Herbert's lab sub-cloned these constructs into a pNN265 vector that contains rabbit gamma globulin introns and poly A tail. A 2 kb fragment was liberated by a Not1 digestion and inserted behind the 1kb promoter sequence from human CD68 (macrosialin) including the 89 bp intronic enhancer (provided by Peter Murray at St. Jude Hospital) and confirmed by restriction digest and DNA sequencing. CD68-CA- or DN-AMPKα1 transgenic mice were generated by pronuclear injection of fertilized C57BL/6 oocytes at the University of Cincinnati transgenic core facility.

Macrophage-specific DN-AMPKα1 mice develop spontaneous obesity associated with increased proinflammatory cytokine production

Macrophage-specific CA-AMPKα1 and DN-AMPKα1 transgenic mice were successfully generated in Dr. Herbert's lab and shipped to our lab. A series of characterization studies were performed on these mice before the generation of macrophage-specific CA- or DN-AMPKα1 / LDLR-/- mice. Genomic DNA was isolated from these mice from the tail tissue and was used for genotypic classification. Based on the genotypic classification of these mice, BMDM were generated with the procedure described in the method. Whole cell lysates were collected from the resting BMDM at day 7 of *in vitro* culture for Western blot analysis of basal level AMPK activity. ACC is a substrate of AMPK and its phosphorylation level is generally used as indicator of AMPK regulation. Both phospho-ACC and total-ACC expression level were detected by Western blot. As shown in Fig. 5A, CA-AMPKα1 BMDM expressed elevated p-ACC compared to WT BMDM whereas DN-AMPKα1 BMDM expressed decreased p-ACC compared to WT BMDM, indicating constitutively activated AMPK activity in CA-AMPKα1 mice and dominant negative AMPK expression in CA-AMPKα1 mice. Suprisingly, the F1 offspring of the macrophage-specific DN-AMPKα1 mice developed spontaneous whole body obesity after 16-20 weeks of age under the animal care facility in Baxter Research Center, University of Louisville (Fig. 5B). The macrophage-specific CA-AMPKα1 mice and WT mice at the same age under the same animal care facility did not develop such phenotype. Fatty liver was associated with the spontaneous obese phenotype of the macrophage-specific DN-AMPKα1 mice, and the expression of both A-FABP and E-FABP were dramatically increased in Kupffer cells isolated from the fatty

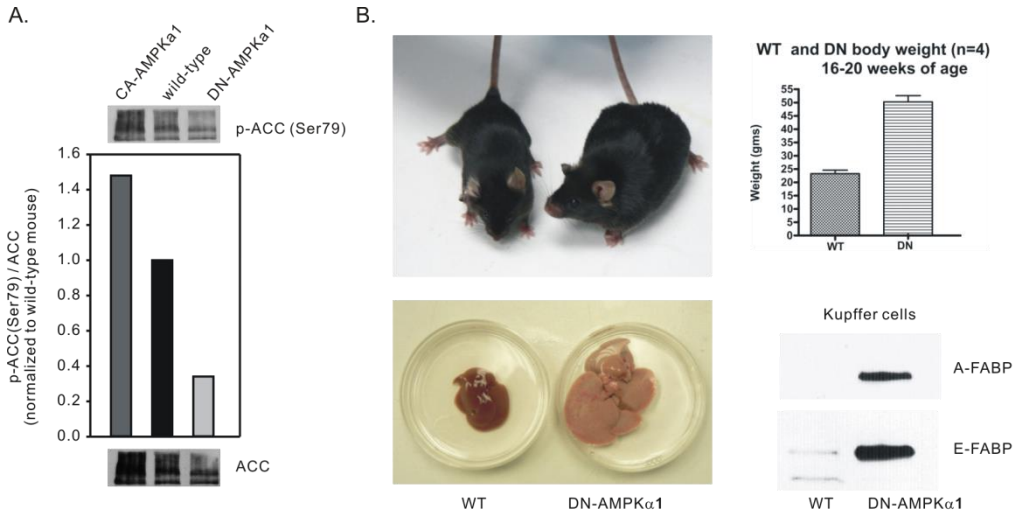

Figure 5. Macrophage-specific DN-AMPKα1 mice develop spontaneous obesity. (**A**), Bone marrow derived macrophages (BMDM) were generated from macrophage-specific CA- or DN-AMPKα1 and wild type (WT) control mice. Whole cell lysates were collected from the resting BMDM at day 7 of *in vitro* culture for Western blot analysis of both p-ACC and t-ACC detection. The ACC phosphorylation level is analyzed by densitometry and shown as bar histogram. (**B**), Photos were taken to show the weight difference between a WT and a DN-AMPKα1 mouse at 2 months of age. Average body weight (n=4) was shown as bar histograms between the two groups. Kupffer cells were isolated from the livers of these mice and whole cell lysate were generated for Western blot detection of A-FABP and E-FABP.

Genotype	mouse #	weight (g)	lungs (g)	spleen (g)	1 kidney (g)	liver (g)	retroperitoneal fat (g)	epididymal fat (g)	inguinal fat (g)	brown fat (g)
WT	937		0.1	<0.1	0.1	1.4	<0.1	0.4	0.3	<0.1
WT	934		0.2	0.2	0.2	1.6	<0.1	0.4	0.2	<0.1
DN-AMPKα1	B0124		0.2	0.1	0.3	**4.1**	**1.6**	**2.0**	**1.5**	0.3
DN-AMPKα1	B0125		0.2	0.1	0.2	**3.5**	**2.4**	**1.9**	**2.1**	

Genotype	mouse #	weight (g)	lungs (g)	spleen (g)	1 kidney (g)	liver (g)	retroperitoneal fat (g)	epididymal fat (g)	inguinal fat (g)	brown fat (g)
WT	1	24.3				1.2	<0.1		<0.1	<0.1
WT	2	21.9				1.3	<0.1		0.1	<0.1
WT	3	22.7				1.2	<0.1		<0.1	<0.1
DN-AMPKα1	A305	29.7				**1.6**	**0.7**	1.7	**0.8**	<0.1
DN-AMPKα1	A306	29.2				**1.4**	**0.6**	1.4	**1.0**	0.1
DN-AMPKα1	A304	29.5				**1.6**	**0.9**	1.4	**0.9**	0.1
CA-AMPKα1	B0193	16.6				1.0	<0.1		<0.1	<0.1
CA-AMPKα1	B0194	18.2				1.1	<0.1		<0.1	<0.1
CA-AMPKα1	B0190	17.5				1.0	<0.1		<0.1	<0.1

Table 1. Macrophage-specific DN-AMPKα1 mice develop whole body obesity associated with fatty liver.

liver of macrophage-specific DN-AMPKα1 mice (Fig. 5B). We further harvested organs and fat tissues from different parts of the body from age-matched WT, macrophage-specific CA-AMPKα1 or DN-AMPKα1 mice and compared their weights. Differences in the weight of the organs and fat tissue between groups were shown in Table 1 (T. 1). Compared to WT and macrophage-specific CA-AMPKα1 mice, the weight of the liver and fat tissue isolated from macrophage-specific DN-AMPKα1 mice were higher (T. 1).

Further characterization on these mice was performed by the evaluation of cytokine production in macrophages. LPS-induced cytokine production in BMDM generated from these mice was measured by ELISA. Expression of the proinflammatory cytokine, IL-6, was decreased in macrophages isolated from macrophage-specific CA-AMPKα1 mice, whereas its expression was elevated in DN-AMPKα1 macrophages compared to in WT macrophages (Fig. 6). The same expression pattern was also seen with TNFα and MCP-1. These data suggest that the expression of dominant negative form of AMPKα1 in macrophages promotes a proinflammatory state in these transgenic mice.

ABCA1 expression is increased in macrophages from macrophage-specific CA-AMPKα1 mice

The primary data we collected from the macrophage-specific CA- or DN-AMPKα1 transgenic mice clearly suggested an import role of macrophage-expressed AMPK in the control of body weight growth and inflammatory cytokine productions. Unfortunately, the F2 offspring of macrophage-specific DN-AMPKα1 mice lost the

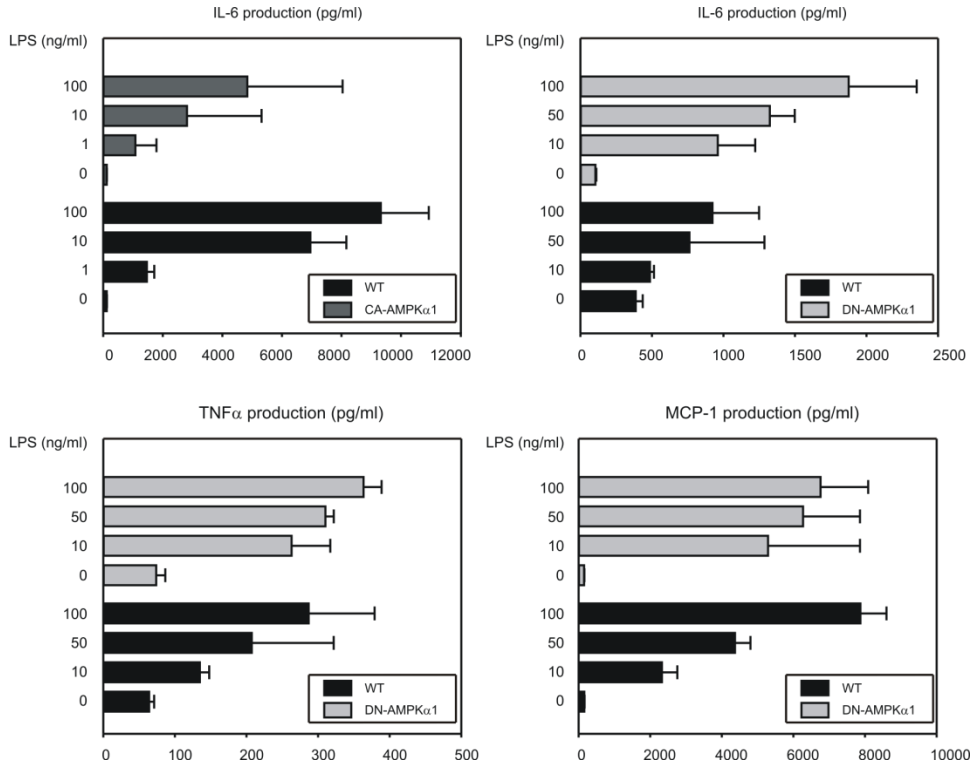

Figure 6. Macrophage-specific DN-AMPKα1 mice are inflammation-prone compared to WT and CA-AMPKα1 mice. Bone marrow derived macrophages (BMDM) generated from macrophage-specific CA- or DN-AMPKα1 and wild type (WT) control mice were incubated with LPS (10, 50, 100 ng) overnight. Supernatants were collected for ELISA analysis of inflammatory cytokine production. Data shown are mean ± SEM of triplicate determinations.

spontaneous obese phenotype after the animals were transferred from the animal care facility in Baxter Research Center, University of Louisville to the animal care facility in Medical Dental Research (MDR) building, University of Louisville. Additionally, in the process of crossbreeding between the macrophage-specific CA- or DN-AMPKα1 with LDLR-/-mice, the F1 offspring of these mice had lost the constitutively active or dominant negative expression of AMPKα1 activity in the macrophages isolated from bone marrow, peritoneal, and spleen. We then put the macrophage-specific CA- or DN-AMPKα1 and WT mice on a diet with 60% cal fat to test if the diet induced differential obesity development between the different genotypes of mice. The body weights of these mice were measured every two days and were shown in Fig. 7A. The organs and fat tissue were harvested from these mice and weighed between 70-80 days of HFD treatment (Table. 2). Fat associated macrophages (FAM) were isolated from the fat tissue and were subjected to RT-PCR analysis of ABCA1 expression. ABCA1 belongs to the family of ATP-binding cassette (ABC) transporters, and enhances cholesterol trafficking in macrophages and prevent atherosclerosis development (154, 174). Deletion of ABCA1 in macrophages enhances atherosclerosis in mice (175). Although the differences of the diet-induced obesity degree were not dramatically apparent as the spontaneous obese phenotype we observed in the macrophage-specific DN-AMPKα1 F1 offspring (Fig. 5 and 7A), the macrophage-specific DN-AMPKα1 mice fed on HFD still possess higher fat tissue distribution all over the body compared to the macrophage-specific CA-AMPKα1 and WT mice (Table. 2). Interestingly, the FAM isolated from macrophage-specific CA-AMPKα1 fed on HFD expressed significantly elevated ABCA1 (Fig. 7B), indicating increased cholesterol efflux in these macrophages. A consistent

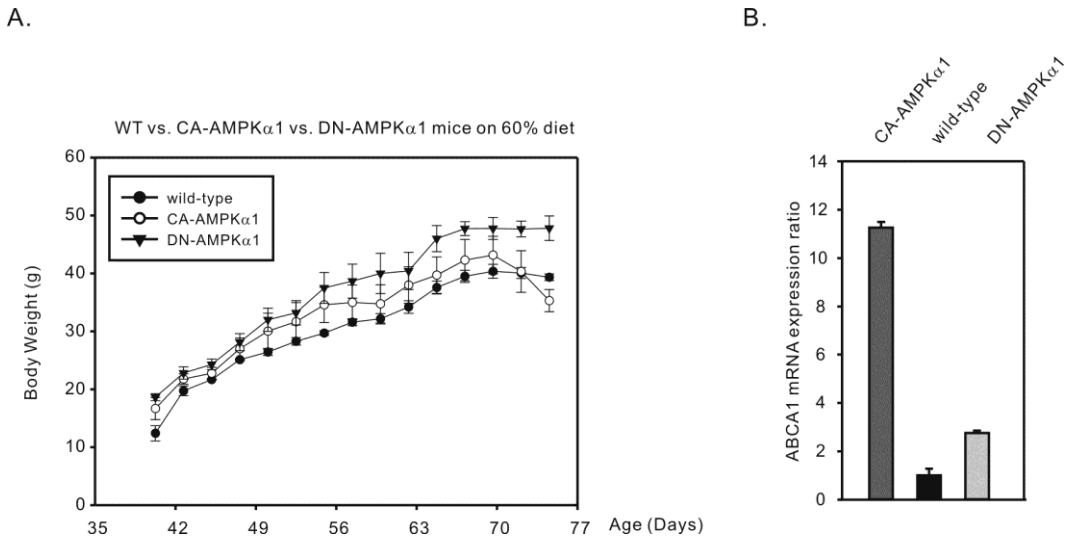

Figure 7. ABCA1 expression is increased in CA-AMPKα1 mice. Macrophage-specific CA-AMPKα1 and DN-AMPKα1 transgenic mice with C56BL/6J control mice were placed on the diet for 80 days. (**A**), Whole body weights of individual mice were assessed every two days. Average body weight was shown as line histogram (n=10). (**B**), Fat tissue was isolated from different parts of the body and weighed (weight shown in Table 2) before FAM isolation. FAM were isolated from the fat tissue from these mice and were subjected to RT-PCR analysis of ABCA1 expression. ABCA1 mRNA expression level was normalized to β-actin. Data shown are mean ± SD of triplicate determinations.

Days of HFD	mice genotype	mouse #	Weight (g)	Total Fat (g)	SVT Cell #	SVT:TF (#cells/g)	heart (g)	spleen (g)	liver (g)	Retroperitoneal Fat	Epididymal Fat	Inguinal Fat	Brown Fat
70	CA4	B982	40.5	9.9	12.4m	1.25m/g	0.1	0.1	1.8	7.9	1.2	0.2	0.6
	DN5	B903	41.2	**11**	**17.2m**	1.56m/g	0.2	<0.1	1.5	8.6	1.7	0.1	0.6
	WT	B956	40	10.2	18.5m	1.81m/g	0.2	0.1	1.2	8.1	1.5	0.1	0.6
80	CA4	B980	48.4	11.7	7.1m	0.61m/g	0.2	0.2	2.5				3.3
	DN5	B899	47.1	**14.1**	**17.1m**	**1.21m/g**		0.1	2.4	9	1.9	0.3	1.6
	WT	B949	49.3	12.8	6.2m	0.48m/g		0.1	2.3	12.5	1.6		0.6

Table 2. Fat tissue weight chart of the macrophage-specific CA- or DN-AMPKα1 and WT mice fed on HFD.

atheroprotective role of AMPK was observed in the subsequent mechanistic studies.

AMPK enhances atheroprotective gene expression induced by IL-10 in macrophages

While the unstable phenotype of the macrophage-specific CA- or DN-AMPKα1 transgenic mice slowed down our research with the atherosclerosis disease model, in the meantime we found an interesting role of AMPK in regulating atherosclerosis-related signaling pathways in macrophages. IL-10 is an important antiinflammatory/ atheroprotective cytokine regulating macrophage antiatherogenic functions in the way of promoting cholesterol trafficking via induction of PPARγ-mediated expression of atheroprotective genes such as ABCA1, apoE, and LXRα, thereby inhibiting proinflammatory gene expression (132). Other IL-10-induced genes include the antiinflammatory proteins such as SOCS family members which serve as effective counter-regulators to inflammatory TLR and cytokine stimuli, as well as Arg1, TGFβ and IL-10 itself (176). IL-10 is also a potent activator of AMPK in macrophages (14). To assess the role of AMPK in the regulation of macrophage functions in atherosclerosis, we examined expression of a panel of genes induced by IL-10 treatment at different time points post-stimulation (30 min - 18 h) in BMDM generated from AMPKα1+/+ and AMPKα1-/- mice. As shown in Fig. 8, as expected, IL-10 stimulation increased mRNA expression of genes encoding apoE, SOCS1, SOCS3, LXRα, ABCA1, IL-10 and TGFβ in AMPKα1+/+ macrophages to varying degrees (1.2 to ~ 10-fold, peak expression time point is shown for each gene). However, in AMPKα1-/- macrophages, IL-10 induction of expression of these genes was either abrogated (e.g., apoE, SOCS3, SOCS1, LXRα, ABCA1) or reduced (Arg1). IL-10 induction of IL-10 and TGFβ mRNA expression in

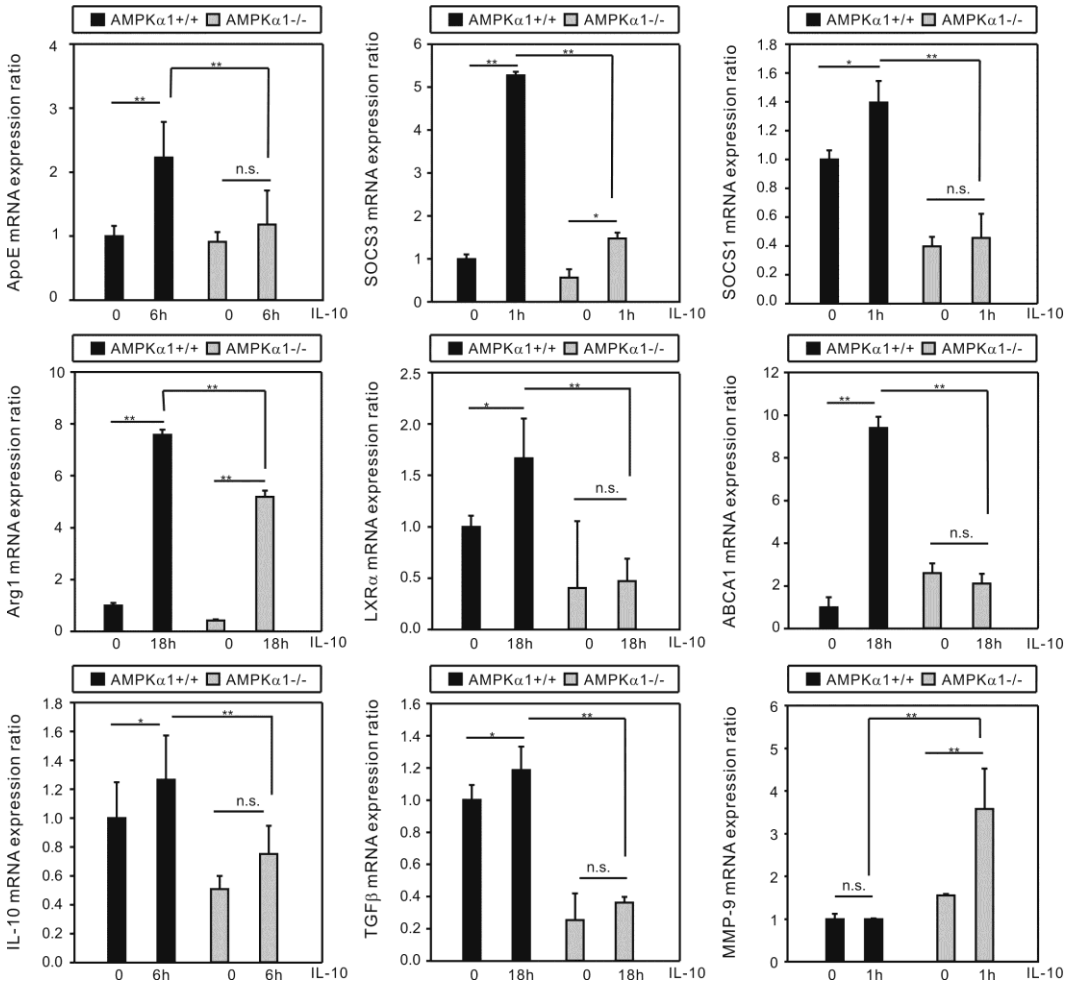

Figure 8. AMPK enhances atheroprotective gene expression induced by IL-10 in macrophages. BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with recombinant mouse (rm)-IL-10 (20 ng/ml) for the duration of 30 min, 1 h, 3 h, 6 h, and 18 h. Total cellular lysates were collected for real-time PCR analysis. The time point representing the peak level of expression for each gene is shown (representative result of two or more independent experiments). Data shown are the mean ± SD of triplicate determinations.

AMPKα1+/+ macrophages was modest (1.3 and 1.2 –fold respectively), however, interestingly, levels of expression in AMPKα1-/- macrophages were below the baseline levels of AMPKα1+/+. We also noted that macrophage-derived MMP-9, which belongs to a family of protease-activated enzymes that can degrade various types of ECM proteins that has critical macrophage-mediated roles in atherosclerosis (177), was upregulated by IL-10 only in AMPKα1-/- macrophages, suggesting a counter-regulatory role of AMPK in MMP-9 expression.

AMPK promotes apoE expression in macrophages

ApoE is an important determinant of atherosclerosis and positive regulator of cholesterol efflux in macrophages. Macrophages isolated from apoE-/- mice express markedly lower cholesterol efflux (178), and transplantation of bone marrow derived from apoE null mice resulted in a significant increase in atherosclerotic lesion size compared to controls in C57BL/6 mice (179). Conversely, transplantation of bone marrow from wild-type mice into apoE-null mice slowed the progression of atherosclerosis (180, 181). These researches suggest that myeloid expression of apoE is critical to attenuate atherosclerosis initiation and progression. Interestingly, as is the case for AMPK (14), apoE has also been recognized as a regulator of macrophage functional plasticity that promotes an antiinflammatory phenotype (182, 183). IL-10 and PPARγ are suggested responsible for apoE expression in macrophages (133, 135). IL-10 induced significant increase of apoE expression in macrophages, and this effect was abrogated in macrophages deficient in AMPK expression (Fig. 8), suggesting that IL-10-induced apoE expression depends on AMPK.

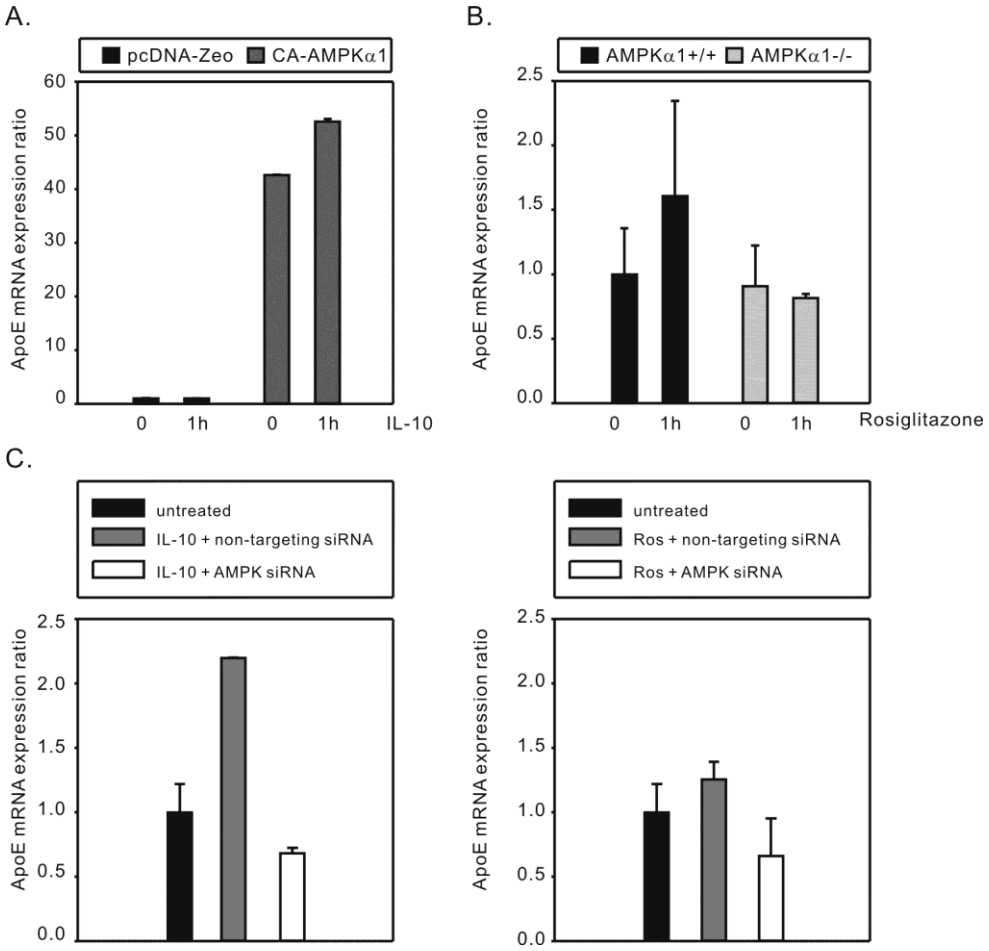

Figure 9. AMPK promotes ApoE expression in macrophages. (**A**), stable transfected macrophage cell line that express CA-AMPKα1 and the control empty vector were generated and treated with recombinant mouse (rm)-IL-10 (20 ng/ml) for 1h. Whole cell lysates were collected for real-time analysis of ApoE expression. (**B**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rosiglitazone (2μM) for 1h. Whole cell lysates were collected for real-time analysis of ApoE expression. (**C**), BMDM generated from C56BL/6J mice were transfected with AMPK siRNA and control non-targeting siRNA. 24h post transfection, 20ng/ml rm-IL-10 or 2μM rosiglitazone were added into the cell culture for 1h stimulation. Cell lysates were analyzed by real-

time PCR for ApoE expression. ApoE mRNA expression levels were normalized to β-actin. Data shown are mean ± SD of triplicate determinations. All the data are from one representative experiment out of two independent experiments with similar results.

This AMPK dependency was further investigated in macrophage cell line that expresses CA-AMPKα1 stimulated with IL-10 and with the use of a PPARγ agonist, rosiglitazone. As shown in Fig. 9A, CA-AMPKα1 macrophages express remarkably elevated apoE (~60-fold) at both basal levels and with IL-10 stimulation compared to the control cell line. Similar to IL-10 stimulation, treatment with rosiglitazone resulted in increased apoE mRNA transcription about 2-fold 1h post stimulation, and this effect was abrogated in AMPKα1-/- macrophages (Fig. 9B). Likewise, silencing of AMPK expression with siRNA transfection led to inability for both IL-10 and rosiglitazone to induce apoE expression. These data support a critical role of AMPK in mediating apoE expression in macrophages, and this AMPK-dependency may contribute greatly in the regulation of macrophage function in inflammatory responses as well as cholesterol trafficking that both play important roles in atherosclerosis.

Relationship between PPARγ and AMPK in macrophages

In our hypothesis, a mechanistic link between FABP/PPARγ/IL-10/AMPK is suggested as the underlying mechanism of AMPK regulation of macrophage atherosclerosis-related functions. To test this hypothesis, a variety of PPARγ agonists including 13-HODE, 15dPGJ2, rosiglitazone and antagonist such as GW9662 was used to activate or block PPARγ activity and the effect on AMPK expression and activation was evaluated. PPARγ expression was sufficiently silenced by 24h siRNA transfection in Fig. 10A, whereas AMPK expression was not affected by this treatment, indicating that PPARγ expression itself does not affect AMPK expression. However, inhibition of PPARγ activity by 24h co-treatment with PPARγ antagonist GW9662 and LPS repressed

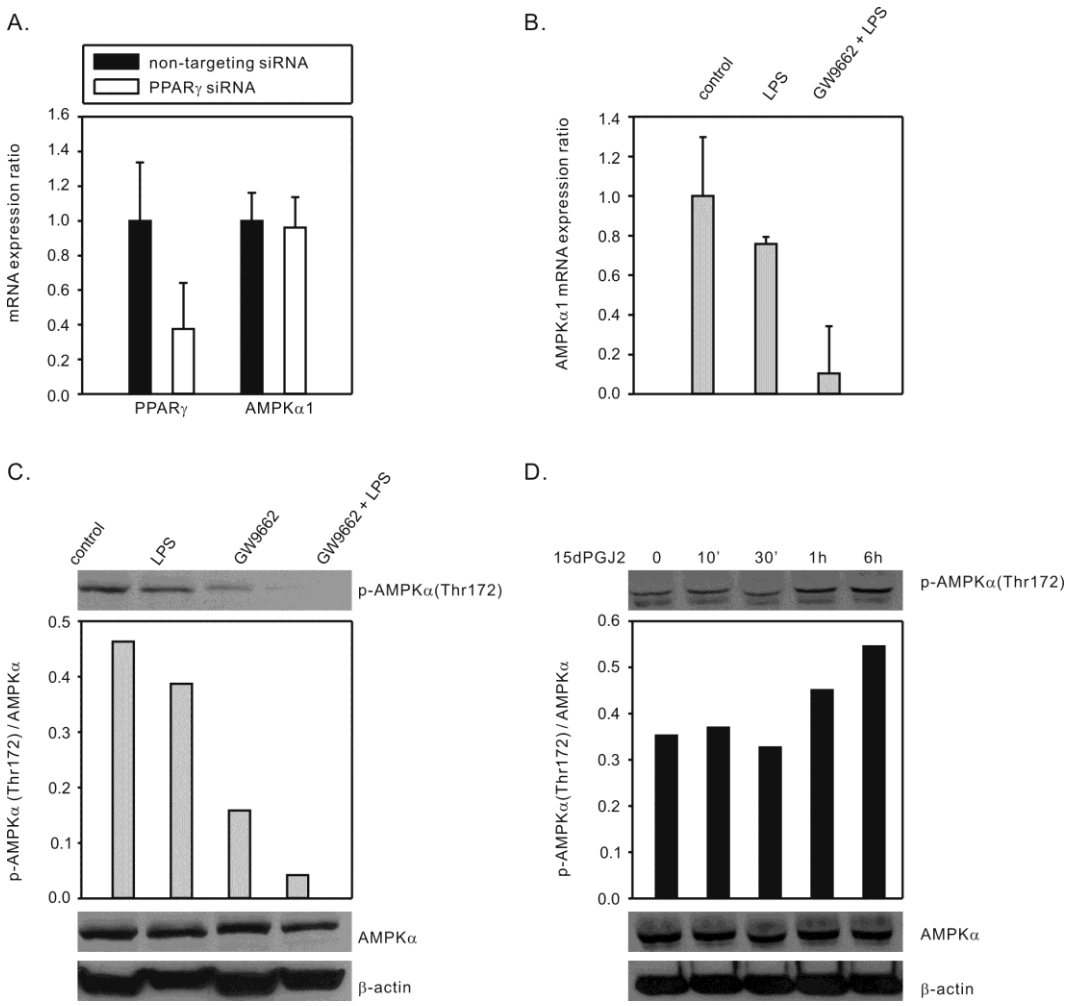

Figure 10. PPARγ activity positively regulates AMPK. (**A**), BMDM generated from C56BL/6J mice were transfected with PPARγ siRNA or control non-targeting siRNA. Whole cell lysates were collected 24h post transfection for real-time analysis of PPARγ and AMPKα1 expression. (**B**), BMDM generated from C56BL/6J mice were treated with LPS (100ng/ml) alone, or LPS (100ng/ml) with GW9662 (2μM) for 24 h. Cell lysates were analyzed by real-time PCR for AMPKα1 expression. (**C**), BMDM generated from C56BL/6J mice were treated with LPS (100ng/ml) alone, or LPS (100ng/ml) with GW9662 (2μM) for 12 h. Cell lysates were analyzed by Western blot for AMPKα-Thr172 phosphorylation. (Continued on next page…)

Figure 10, continued. (**D**), BMDM generated from C56BL/6J mice were treated with 15dPGJ2 (3μM). Whole cell lysates were collected at 0, 10', 30', 1h, 6h post stimulation respectively for Western blot assessment of AMPKα-Thr172 phosphorylation. (**A** and **B**), gene mRNA expression levels were normalized to β-actin. Data shown are mean ± SD of triplicate determinations. (**C** and **D**), AMPK activation level was analyzed by densitometry and displayed as bar histogram. The results shown are representative of two or more independent experiments.

the expression of AMPKα1 mRNA and this reduction of expression was about 80-fold higher than LPS alone group (Fig. 10B), indicating that inhibition of PPARγ activity led to reduced AMPK expression in macrophages. This finding is supported by Western blot analysis of AMPK activity in macrophages treated with GW9662 and/or LPS. 12h treatment of LPS resulted a reduction of AMPK activation (Fig. 10C), this result is consistent with the data we showed prevously (14). Compared to the LPS treated group, treatment with GW9662 resulted more potent inhibition on AMPK activation and the cobination of LPS and GW9662 completely abrogated AMPK activation (Fig. 10C). Conversely, 1-6 h treatment of PPARγ agonist 15dPGJ2 resulted AMPK activation in macrophages (Fig. 10D). These data supported our hypothesis that elevated PPARγ activity results in increased AMPK activity in macrophages.

We noticed that the PPARγ agonist 15dPGJ2 activates AMPK at a relatively late time point (6h). This late activation implicates an indirect regulation of AMPK activity mediated by PPARγ inducible gene expression. Because PPARγ promotes IL-10 expression and IL-10 activates AMPK (14, 158), IL-10 is likely the intermediate factor that links PPARγ and AMPK activity. To verify the role of IL-10 in this PPARγ/AMPK regulation, we used a neutralization antibody to block endogenous IL-10 signaling. Before use, the efficacy of this antibody was demonstrated by examination of IL-10-induced signal transducer and activator of transcription 3 (STAT3) activation with anti-IL-10 pretreatment. IL-10 induces rapid STAT3 activation in macrophages (184). 1h pretreatment with anti-IL-10 antibody at concentration of 50-150 ng/ml efficiently blocked IL-10 activation of STAT3 (Fig. 11A). Like 15dPGJ2, stimulation with PPARγ agonist 13-HODE induced AMPK activation at 6 h time point, and pretreatment with

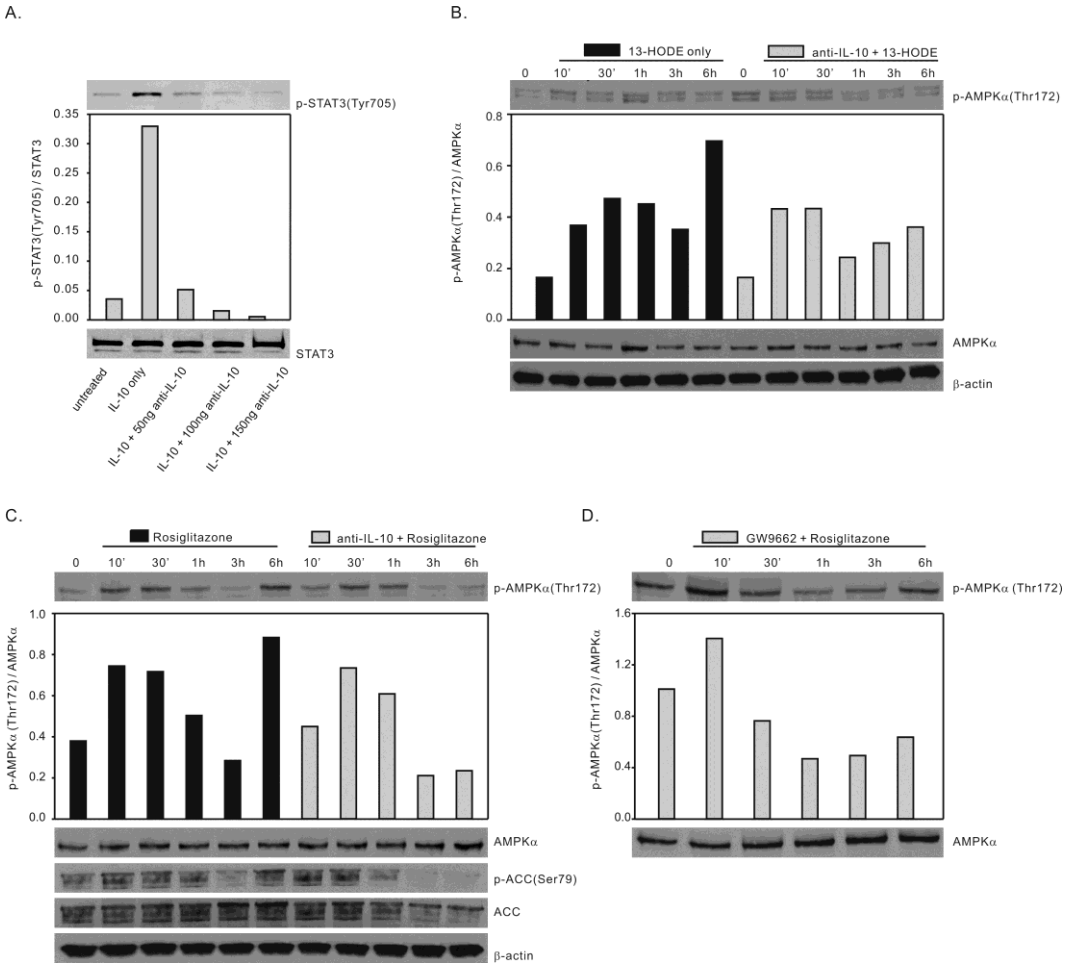

Figure 11. PPARγ agonists induce AMPK activation and require IL-10 expression. (**A**), BMDM generated from C56BL/6J mice were treated with anti-IL-10 neutralization antibody (50, 100, 150 ng/ml) 1h pre-incubation, then exposed to rm-IL-10 (20ng/ml) stimulation for 1h. Cell lysates were analyzed by Western blot for STAT3-Tyr705 phosphorylation. (**B**), BMDM generated from C56BL/6J mice were treated with anti-IL-10 neutralization antibody (100 ng/ml) 1h pre-incubation, then exposed to 13-HODE (10μM) for indicated time. Cell lysates were analyzed by Western blot for AMPK-Thr172 phosphorylation. (**C**), BMDM generated from C56BL/6J mice were treated with anti-IL-10 neutralization antibody (100 ng/ml) 1h pre-incubation, then exposed to rosiglitazone (2μM) for indicated time. (Continued on next page…)

Figure 11, continued. Cell lysates were analyzed by Western blot for AMPK-Thr172 and ACC-Ser79 phosphorylation. (**D**), BMDM generated from C56BL/6J mice were treated with GW9662 (2μM) for1h pre-incubation, then exposed to rosiglitazone (2μM) for the indicated times. Cell lysates were analyzed by Western blot for AMPK-Thr172 phosphorylation. Protein activation levels were analyzed by densitometry and displayed as bar histogram. The results shown are representative of two or more independent experiments.

anti-IL-10 neutralization antibody abolished this effect (Fig. 11B). A similar effect was also seen upon stimulation with another PPARγ agonist, rosiglitazone, without or with IL-10 neutralization (Fig. 11C). Interestingly unlike 15dPGJ2 induced AMPK activation, an early activation of AMPK between 10 min to 1 h was observed with stimulation by 13-HODE or rosiglitazone (Fig. 11B and C). To test if this early activation of AMPK by PPARγ agonists was mediated by PPARγ activity, we pretreated BMDM with GW9662 to block PPARγ activity followed by stimulation with rosiglitazone. The early AMPK activation stimulated by rosiglitazone was not affected by GW9662 pretreatment (Fig. 11D), suggesting this early AMPK activation by PPARγ agonist is independent of PPARγ activity. These data demonstrated that PPARγ activity positively regulates AMPK activity in macrophages, and this regulation requires IL-10 signaling.

An interesting result was observed during the research of PPARγ/AMPK relationship. We noticed that silencing of AMPK expression by siRNA also suppressed PPARγ expression (Fig. 12A), in contrast to the observation that PPARγ siRNA treatment did not affect AMPK expression (Fig. 10A), and suggested a positive regulation of PPARγ expression by AMPK. This conclusion is supported by the data we collected from the CA-AMPKα1 macrophage cell line, which showed an elevated PPARγ expression at both baseline and IL-10 stimulated level (Fig. 12B).

Our data showing that PPARγ activity positively regulates AMPK activation via IL-10 production, and AMPK in turn, regulates PPARγ expression, indicated that there is a co-dependent relationship between PPARγ and AMPK in macrophages.

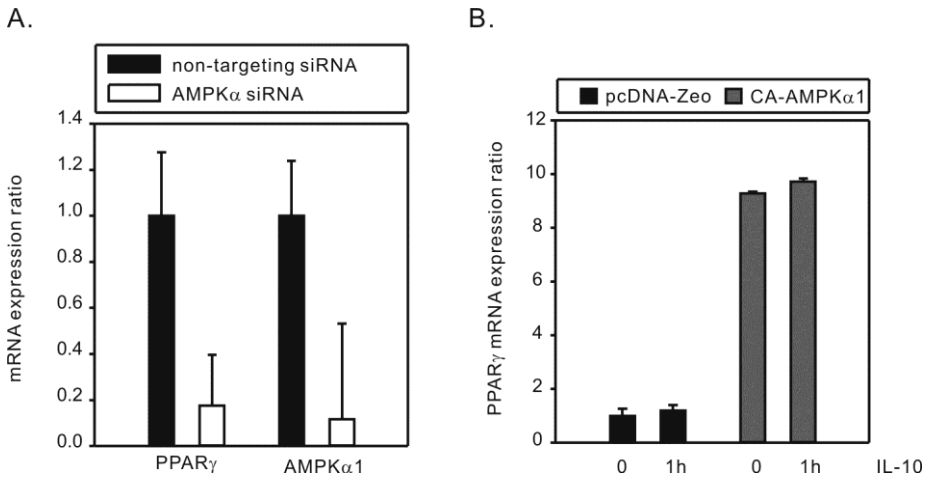

Figure 12. AMPK positively regulates PPARγ expression. (**A**), BMDM generated from C56BL/6J mice were transfected with AMPK siRNA and control non-targeting siRNA. Whole cell lysates were collected 24h post transfection for real-time analysis of PPARγ and AMPKα1 expression. (**B**), CA-AMPKα1 and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for 1 h. Whole cell lysates were collected for real-time analysis of PPARγ expression. Data shown are mean ± SD of triplicate determinations. All the data are one representative experiment out of two independent experiments with similar results.

DISCUSSION

Macrophages are key players in atherosclerosis. They perform a variety of regulatory functions including lipid trafficking, energy homeostasis, and inflammatory responses that are closely related to every step of atherogenesis from lesion formation to plaque destabilization. The physiological microenvironment within atherosclerosis lesion contains diverse factors with opposing influences of inflammation properties, such as IFNγ or IL-10, that have the potential to promote macrophage functional adapt towards either atherogenic or antiatherogenic directions, respectively. Our research has established a key regulatory role of AMPK in macrophage inflammatory functions (14). Antiinflammatory stimulation with IL-10 activates AMPK, thus turns on the signaling events that eventually lead to suppression of NF-κB mediated proinflammatory cytokine productions while enhancing IL-10 production.

The primary data we collected from the macrophage-specific CA-AMPKα1 and DN-AMPKα1 transgenic mice provided valuable evidence to support a counter regulatory role of macrophage expressed AMPK in the development of metabolic syndromes including obesity and atherosclerosis. Transgenic mice that expressed a dominant negative form of AMPK specifically in their macrophages had more inflamed macrophages and were more susceptible to obesity, even without intake of excessive dietary fat (Fig. 5 and 6). On a HFD, these mice developed excessive fat all over the body while in the contrast the transgenic mice that expressed macrophage-specific CA-

AMPKα1 showed much higher levels of ABCA1, indicating accelerated cholesterol trafficking in their fat associated macrophages (Fig. 7). Although the instability of the phenotypes of these transgenic mice caused an unexpected gap for this research, a mechanism demonstrating a co-dependent relationship between PPARγ and AMPK in macrophages provided futher evidence to support the beneficial role of AMPK in atherosclerosis (Fig. 10-12).

More importantly, a requirement of AMPK for the potent antiinflammatory/ antiatherogenic cytokine IL-10 to induce atheroprotective gene expression including ABCA1, apoE, LXRα, and IL-10 itself, was discovered. This discovery is important due to the broad-reaching significance of IL-10 in maintaining immune homeostasis, and in the regulation in many inflammatory diseases including obesity, atherosclerosis, autoimmune disease, and cancer. The investigation of the role of AMPK in IL-10 mediated signaling events that eventually led to genetic modification of macrophage functions may provide valuable insights into therapies of a broad range of inflammatory diseases including atherosclerosis as well as the understanding of IL-10's suppressive functions to maintain immune homeostasis in the body. Experiments were then carefully designed for this purpose, and the results are shown and discussed in the next chapter.

CHAPTER 3

AMPK REGULATES IL-10-MEDIATED ANTIINFLAMMATORY SIGNALING PATHWAYS IN MACROPHAGES

INTRODUCTION

In the process of investigating of the regulatory mechanisms of AMPK in macrophage functional polarization in atherosclerosis, we found that AMPK is essential for IL-10-induced expression of genes including these encoding apoE and suppressor of cytokine signaling 3 (SOCS3) (Fig. 8). In addition to AMPK (14), ApoE is also recognized as a regulator of macrophage plasticity that promotes antiinflammatory phenotype (182, 183). SOCS3 is an established inhibitory protein rapidly induced by IL-10, and potently inhibits inflammatory responses in many cell types including macrophages (185, 186). Our discovery of a requirement of AMPK for IL-10-mediated gene expression led us to re-focus efforts on the role of AMPK in IL-10 signal transduction. In doing so, an essential role of AMPK in IL-10-mediated antiinflammatory signaling and function was evident by data demonstrating a requirement of AMPK in IL-10 activation of the PI3K/Akt/mTOR and STAT3/SOCS3 pathways. The important role of AMPK in maintaining energy homeostasis has been

well studied in non-immune cells such as endothelial cells and SMCs. Recent studies revealed the importance of leukocyte AMPK in the immune suppression of inflammation in health and disease (187). This study of AMPK regulation of IL-10-mediated antiinflammatory signaling pathways and functions in macrophages is an important addition to the understanding of AMPK biological functions and the underlie mechanisms of the suppressive immune surveillance, therefore provides the fundamental molecular mechanisms for the treatment of inflammation-associated diseases including atherosclerosis.

AMPK signaling

The role of AMPK in mediating metabolic pathways in the body is well studied. Recent work has also established an association of AMPK activity with inflammatory potential in leukocytes (11, 13-15, 111). For example, silencing of AMPKα1 in macrophages or forced expression of a dominant negative form of AMPKα1 results in amplification of inflammatory activity, whereas treatment with agents that activate AMPK is suppressive (14). Stimulation of macrophages with proinflammatory agents such as LPS reduces AMPK phosphorylation, whereas stimulation with antiinflammatory agents (e.g., IL-10, IL-4 and TGFβ) results in elevated levels of phospho-AMPK (14). Likewise, TLR activation by LPS in DCs induced decreased AMPK activation and this effect was accompanied by increased cellular glucose consumption (110). We demonstrated recently that AMPK deficiency in DC results in a heightened inflammatory response to CD40 stimulation, resulting in an increased capacity to induce Th1 and Th17 differentiation during antigen presentation (15). AMPKα1 activity was also found to be

responsible for T cell immunoglobulin- and mucin domain-containing molecule-4 (TIM-4)-mediated autophagic degradation of apoptotic tumor cells in macrophages and the subsequent immunosuppressive phenotype macrophages acquire during this process (13). In addition to the discovery of upstream regulators including LKB1, CaMKKβ, and Tak1 kinase (42), identified mechanisms of AMPK-induced suppression of inflammatory signaling in macrophages include activation of inhibitory PI3K-mediated pathways (14) and enhancement of SIRT1 activity and expression leading to NF-κB deacetylation (109).

Studies focusing on AMPK regulation of metabolic pathways in leukocytes have contributed to the emergent paradigm that increased glycolysis is associated with inflammatory activity and proliferation, whereas reduced glycolysis and enhanced oxidative metabolism is associated with suppressed inflammation and quiescence (11). However, activators of AMPK not only suppress inflammatory responses but re-polarize leukocytes by actively inducing programs of antiinflammatory gene expression. The observation that antiinflammatory cytokines, including IL-10, induce rapid activation of AMPK in macrophages (14) suggests that AMPK serves as an immediate upstream signaling molecule in antiinflammatory pathways.

IL-10 signaling

IL-10 is a potent immunosuppressive cytokine that systemically limits excessive inflammation and controls inflammatory disease progression (160, 161). The antiinflammatory function of IL-10 has been well studied in murine models of inflammatory disease including inflammatory bowel disease (IBD), experimental allergic encephalomyelitis (EAE), and atherosclerosis (166, 188, 189). However, the signaling

pathways activated through IL-10 receptor ligation have received less attention. Two important signaling cascades have been reported to mediate IL-10 suppressive functions in macrophages, the PI3K/Akt and JAK/ STAT3 pathways (184, 190).

IL-10 activates PI3K/Akt pathway

IL-10 is reported to promote PI3K/Akt activation. Pharmaceutical inhibition of PI3K in macrophages impairs IL-10-induced gene expression, as well as IL-10-mediated suppression of LPS-induced proinflammatory gene expression and this effect is reversed by the expression of a constitutively active Akt (190). Although PI3K and Akt activity can be induced by IL-10 stimulation in pro-myeloid cells (191), the direct activation of PI3K by IL-10 in macrophages has not yet been reported. Class IA PI3Ks are composed of a catalytic subunit (p110α, p110β, or p110δ) and a tightly associated regulatory subunit (p85α, p85β, p55γ, p55α, or p50α). The regulatory p55 subunit results from the alternate transcription of the *pik3r1* gene (192-194). It associates with the catalytic subunit p110 and coordinates unique PI3K kinase functions such as the induction of cell cycle arrest (195, 196) and prevention of xenograft tumor growth (197). Physiological stimulation such as IL-10 induces phosphorylation of the regulatory subunit of PI3K and results in PI3K activation. Once activated, PI3K converts PtdIns(3,4)P2 (PIP2) into PtIns(3,4,5)P3 (PIP3), and promotes the accumulation of PIP3 on plasma membrane, which leads to colocalization of phosphoinositide-dependent protein kinase 1 (PDK1) and Akt to the plasma membrane where PDK1 phosphorylates Akt directly on Thr308 (198-200). In contrast, phosphatase and tensin homologue deleted on chromosome ten (PTEN) reverses PI3K action by catalyzing dephosphorylation of PIP3 and convert PIP3 into PIP2 (201).

Phosphorylation on three phosphorylation sites, serine-380, threonine-382 and -383 (Ser380/Thr382/383) maintains PTEN at a more stable state of expression (202). PDK1 is constitutively active in cells with different sites phosphorylated in the absence of stimulation, yet so far only serine-241 (Ser241) is proved to be essential for PDK function (199, 203). Phosphorylation of both Thr308 in the activation loop and Ser473 in the kinase tail enhances the activation of Akt (198).

Activation of Akt promotes mTORC1 and GSK3β/CREB activation

Activation of Akt mediates many downstream biological events including the direct activation of mammalian target of rapamycin (mTORC1) (204). Relevant to the work presented herein, it has been reported that IL-10 stimulation of primary monocytes results in elevated mTORC1 activity and this effect is abrogated by PI3K inhibition (205). IL-10 prevents LPS-induced proinflammatory gene expression by the inhibition of NF-κB activation (206) via the PI3K/Akt/GSK3β pathway (190). Phosphorylation/ inactivation of glycogen synthase kinase-3 β (GSK3β) promotes the competitive binding of the nuclear coactivator CBP to cAMP response element-binding protein (CREB), therefore negatively regulates NF-κB activity (207). Interestingly, an inhibitory role of AMPK was observed in IκB degradation and NF-κB activation, and CA-AMPKα1 macrophages express increased CREB activation in response to LPS stimulation (14).

IL-10 activates JAK/STAT3/SOCS3 pathway

Although the precise mechanism of activation of PI3K/Akt signaling via IL-10 in macrophages remains unclear, the direct association of IL-10R with Janus family of

tyrosine kinases (JAKs) and the subsequent phosphorylation/activation of STAT3 and STAT3-directed expression of SOCS3 is well-established (184, 208-211). In addition to the tyrosine phosphorylation events in this pathway, phosphorylation on serine residues within the IL-10 receptor intracellular domain and within STAT3 homodimer complexes are also important for IL-10/STAT3 suppressive function (212-214). Interestingly, mTORC1 was reported to mediate STAT3 serine phosphorylation in different cell types and data suggests that this serine phosphorylation is required for optimal activation of STAT3 (215-218). Following STAT3 activation, the rapid expression of SOCS3 protein potently suppresses TLR inflammation in different cell types including macrophages (185, 219, 220). The mTORC1 protein complex is shown to negatively regulate innate inflammatory responses in myeloid immune cells (221, 222) and, in contrast to non-immune cells in which AMPK typically inhibits mTORC1 (223), evidence suggests that AMPK activity positively regulates mTORC1 activity in macrophages (224).

Given the rapid and robust activation of AMPK in response to IL-10 stimulation, herein we addressed the hypothesis that AMPK orchestrates PI3K/Akt/mTORC1, PI3K/Akt/CREB and JAK/STAT3/SOCS3 signaling pathways to contribute to IL-10-mediated polarization of macrophages to an antiinflammatory phenotype.

MATERIAL AND METHODS

Reagents

Recombinant mouse IL-10 was purchased from R&D Systems. LY294002, JAK inhibitor I, and rapamycin were purchased from Calbiochem (EMD Millipore) and STO-609 was purchased from Tocris Biosciences. The Jak inhibitors AG490 and CP-690, 550 were purchased from LC Laboratories Ltd. Western blot detection of specific proteins used the following primary Abs: anti-phospho-AMPKα (Thr172), anti-AMPKα, anti-phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199), anti-PI3 kinase p55, anti-PI3 kinase p85, anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-GSK3β (Ser9), anti-GSK3β, anti-phospho-CREB (Ser133), anti-CREB, anti-phospho-mTOR (Ser2448), anti-mTOR, anti-phospho-p70 S6K (Ser371), anti-p70 S6K, anti-phospho-TSC2 (Ser939), anti-phospho-TSC2 (Ser1387), anti-TSC2, anti-phospho-Stat3 (Tyr705), anti-phospho-Stat3 (Ser727), anti-Stat3, anti-phospho-JAK1 (Tyr1022/1023), anti-JAK1, anti-SOCS3 (Cell Signaling Technology), anti-β-actin (Sigma-Aldrich), and HRP-conjugated secondary Ab (Jackson ImmunoResearch Laboratories).

Mice and Cell culture

AMPKα1 deficient (AMPKα1-/-) mice were generated as previously described (170). C57BL/6J mice were purchased from the Jackson Laboratory. C57BL/6J mice,

AMPKα1-/- mice and their littermate controls, AMPKα1+/+ mice, were bred and maintained in the Research Resources Facility, University of Louisville. All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee, University of Louisville. Bone marrow-derived macrophages (BMDM) were generated from C57BL/6J mice, AMPKα1+/+ and AMPKα1-/- mice following the protocol previously described (171). BMDM were maintained in incubators set to 37°C and 5% CO_2. For stimuli/reagents used in individual experiments, BMDM were harvested into a 24-well cell culture plate and rested overnight in RPMI medium supplemented with 5% FBS, 10 mM HEPES Buffer (Sigma), and 10 μg/ml gentamycin (Atlanta Biologicals) (referred as R5 medium) prior to stimulus. The cells were quickly rinsed with pre-warmed DPBS supplemented with 2% FBS before lyse.

Western blot analysis

Whole cell lysates were generated by lysis with a buffer containing 125 mM Tris (pH 6.8), 2% SDS, 20% glycerol, 100 μM PMSF, protease inhibitor mixture (Promega), and HALT™ phosphatase inhibitor cocktail (Thermo Scientific). Total protein content of the samples was assessed by BCA protein assay (Pierce). Equal amounts of protein were separated on 10% Criterion gels (Bio-Rad) by SDS-PAGE. Medium and high molecular weight proteins (55-289KDa) were transferred to nitrocellulose membranes using a Trans-Blot® Turbo™ Nitrocellulose Transfer Pack and Trans-Blot®Turbo™ transfer system (Bio-Rad). Low molecular weight proteins (< 55kDa) were transferred to nitrocellulose membranes (Hybond; Amersham Biosciences) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). Ab-bound proteins were detected using

an ECL Western blotting analysis system (Amersham Corp.), and the membranes were exposed to SRX-101A film (Konica Minolta). Densitometry analysis was performed using the UN-SCAN-IT gel (version 6.1) software.

Real-time RT-PCR analysis

mMACs™ One-step cDNA Kit (Miltenyi Biotech) were used for RNA isolation and cDNA synthesis. cDNAs were amplified in a 20 μl reaction volume containing SYBR Green (New England Biolabs) and analyzed using a DNA Opticon 2 Monitor (MJ Research). All the gene mRNA expression levels (*tnfa*, *il-6*, and *il-12b*) were analyzed by Quantitect Primer Assays (Qiagen). The cDNA concentrations in each sample were normalized using transcripts for β-actin. The relative expression software tool (REST©) was used to quantify mRNA expression (172).

ELISA

Following stimulation in 24-well plates, supernatants were collected into 96-well plates and assayed by ELISA using OptEIA™ sets (BD Biosciences Pharmingen) according to the manufacturer's instructions. Analysis was performed using an E-max precision micro plate reader (Molecular Devices).

Statistical Analysis

Statistical significance between groups was calculated with an unpaired Student's *t* test, with a p value < 0.050 considered statistically significant.

RESULTS

IL-10 activates AMPK on a time dependent manner

Our previous research demonstrated a time-dependent activation of AMPK by antiinflammatory factors, including IL-10, in macrophages generated from wild-type mice (14). The influence of IL-10 stimulation on AMPK activation was tested with macrophages generated from both AMPKα1+/+ and AMPKα1-/- mice (Fig. 13). IL-10 stimulation induced rapid AMPK activation in AMPKα1+/+ macrophages within 5 min, as confirmed by both phosphorylation on AMPKα and ACC (Fig. 13A), and this activation persisted as long as the IL-10 stimulation up to > 8 h (Fig. 13B). Both phosphorylated- and total- AMPK expression were wiped out in AMPKα1-/- mice (Fig. 13 A and B). This result revealed a potent role of IL-10 in AMPK activation in macrophages and confirmed the deficient expression of AMPK in AMPKα1-/- mice, which provides a good tool for the studies to investigate the role of AMPK in IL-10 signaling transduction.

AMPK is required for IL-10 activation of PI3K/Akt pathway

The data shown in Figure 8 demonstrate a role of AMPK in IL-10-mediated gene expression, including expression of SOCS3 which has been shown to be largely responsible for the ability of IL-10 to suppress TLR-mediated inflammatory responses (225). We investigated the means by which AMPK signaling contributes to this aspect of

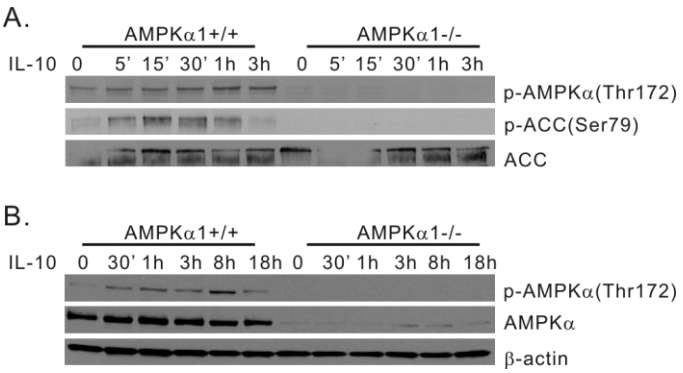

Figure 13. IL-10 activates AMPK in a time-dependent manner. BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for (**A**), AMPKα-Thr172 and ACC-Ser79 phosphorylation and (**B**), AMPKα-Thr172 phosphorylation. The results shown are representative of more than four independent experiments.

IL-10 function. IL-10 is reported to activate the PI3K/Akt pathway in macrophages (190, 191), however direct phosphorylation of PI3K in macrophages through IL-10 stimulation has not been shown. With BMDM generated from AMPKα1+/+ mice, we demonstrated that IL-10 stimulation induced rapid AMPK activation, as indicated by elevated phosphorylation of AMPKα-Thr172 residue and phosphorylation of ACC-Ser79 residue within 5 minutes (Fig. 13A). This effect was accompanied by more than 2 fold increase of the phosphorylation level of the PI3K p55 regulatory subunit within 15 minutes (Fig. 14A). IL-10 induced PI3K activation was not apparent in AMPKα1-/- BMDM (Fig. 14A). Interestingly, we noted that the overall expression level of the PI3K p55 subunit was ~1.5 fold lower in AMPKα1-/- BMDM compared to AMPKα1+/+ BMDM, while the level of p85 subunit expression was similar in both groups. Moreover, elevated PI3K p55 phosphorylation level was observed in CA-AMPKα1 macrophage cell line at both baseline and IL-10 stimulated levels compared to the control macrophages (Fig. 14B). We failed to detect phosphorylated PI3K p85 in both AMPKα1+/+ and AMPKα1-/- BMDM and the transfected macrophage cell lines in repeated experiments, indicating a novel role of IL-10-induced AMPK in the activation of the PI3K p55 regulatory subunit, specifically.

The data in Figure 14 suggested that IL-10 induces transient PI3K activation. The expression of AMPK is required for this effect, and constitutively active AMPK enhances this effect. To test if AMPK is upstream of PI3K activation in IL-10 signal transduction, we pretreated macrophages generated from C57BL/6J mice with the PI3K inhibitor LY294002, then stimulated the cells with IL-10. Cell lysates were collected at both early and late time points (5 min-18 h) for Western blot analysis of both p-Akt and p-AMPK

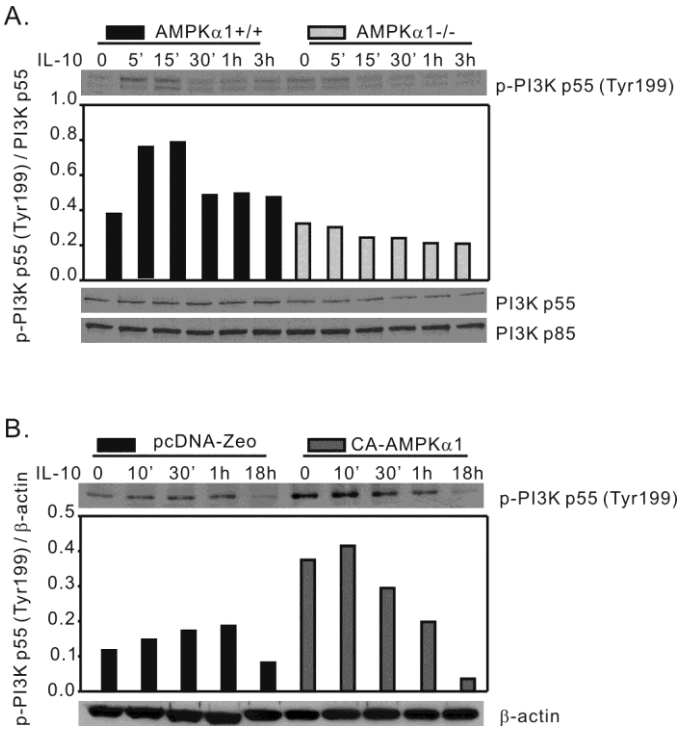

Figure 14. AMPK positively regulates IL-10-induced PI3K activation. (**A**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for p-PI3K p55 (Tyr199), t-PI3K p55, and t-PI3K p85 expression. (**B**), CA-AMPKα1 and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for indicated time. Cell lysates were analyzed by Western blot for PI3K p55-Tyr199 phosphorylation. PI3K activation/ phosphorylation levels were analyzed by densitometry and displayed as bar histogram. The results shown are representative of two or more independent experiments.

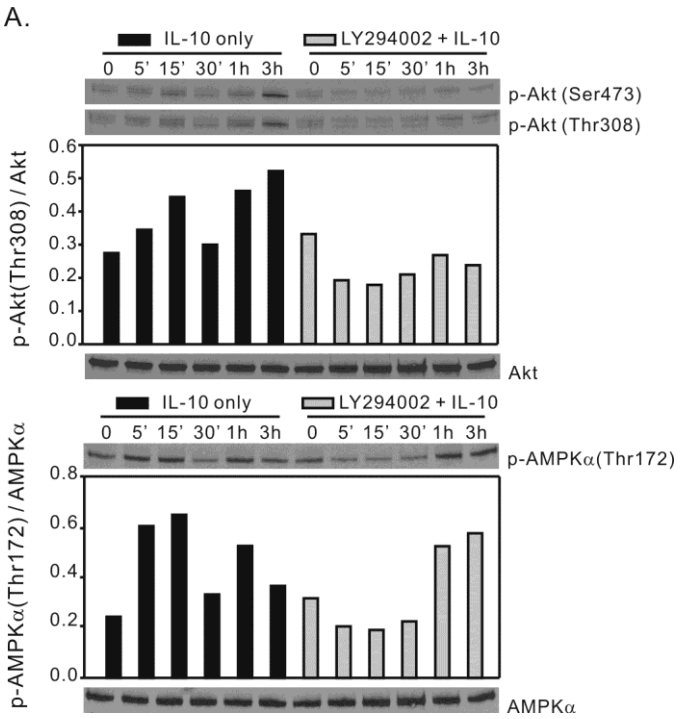

Figure 15. PI3K inhibitor LY294002 does not block IL-10-induced AMPK activation. BMDM generated from C56BL/6J mice were treated with LY294002 (20mM) for 1h pre-incubation, then exposed to rm-IL-10 (20ng/ml) stimulation for indicated time. Cell lysates were analyzed by Western blot for (**A**), Akt-Ser473 and Akt-Thr308 phosphorylation (upper panel), AMPK-Thr172 phosphorylation (lower panel), and (**B**), Akt-Ser473 phosphorylation (upper panel), AMPK-Thr172 phosphorylation (lower panel). (Continued on next page…)

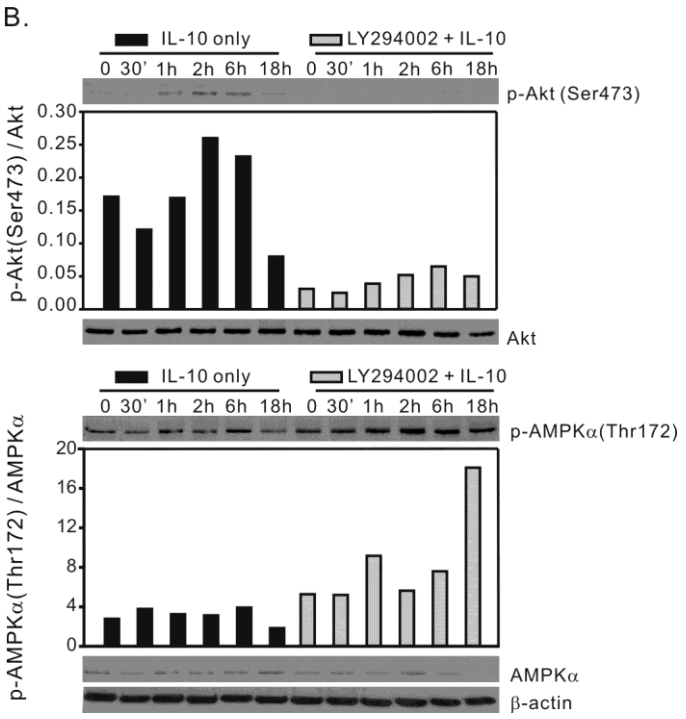

Figure 15, continued. Activation level of proteins were analyzed by densitometry and displayed as bar histogram. The results shown are representative of three independent experiments.

levels. Akt is a downstream target for PI3K phosphorylation and its phosphorylation/ activation was used as an indicator of PI3K inhibition by LY294002. As showed in Fig. 15A, upper panels, pretreatment with LY294002 completely blocked IL-10-induced Akt phosphorylation of both serine and threonine residues, and this was evident throughout a 5'-18h time period (Fig. 15B, upper panels). LY294002 pretreatment resulted in a slight impairment of early phosphorylation of AMPK induced by IL-10 stimulation but phosphorylation returned to control levels after 30 min, and was higher in the LY294002 pretreated macrophages at the later time points (Fig. 15, lower panels). LY294002 is capable of suppressing calcium entry into the cells independent of its inhibition on PI3K activity (226, 227) thus the early inhibition of AMPK by LY294002 may due to LY294002 inhibition of Ca^{2+} influx leading to the inhibition of the AMPK upstream regulator CaM-KKβ (54). These results demonstrate that IL-10 activation of AMPKα1 is upstream of PI3K/Akt signaling in macrophages.

Akt activation is a mutiple-step process that is mediated by several up-stream signaling proteins including PI3K, PTEN, and PDKs. PTEN is a phosphatase that counters PI3K phosphorylation of PIP2, therefore negatively regulates Akt activation. CA- or DN- AMPKα1 macrophages cell lines stimulated with IL-10 indicated a negative role of AMPK in PTEN phosphorylation/expression, as demonstrated in Figure 16: the phosphorylated/activated AMPKα1 is constitutively expressed in CA- AMPKα1 macrophages, whereas in these macrophages the expression of both phosphorylated and total level of PTEN is decreased (Fig. 16A). However PTEN phosphorylation and expression were unaffected in either DN-AMPKα1 macrophages or primary macrophages

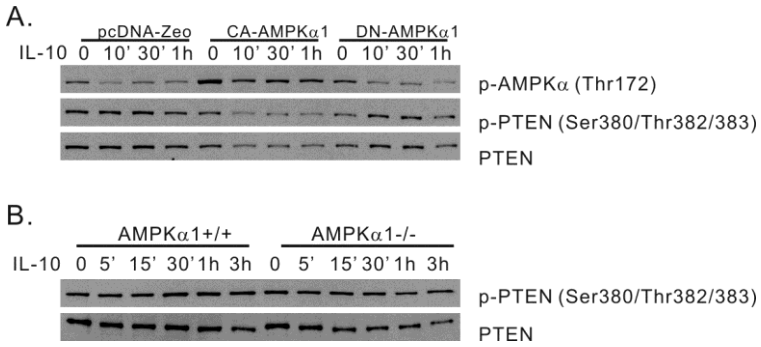

Figure 16. AMPK negatively regulates PTEN activity in response to IL-10. (**A**), CA-AMPKα1, DN-AMPKα1, and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for indicated time. Cell lysates were analyzed by Western blot for AMPKα-Thr172 and PTEN-Ser380/Thr382/383 phosphorylation. (**B**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for PTEN-Ser380/Thr382/383 phosphorylation. The results shown are representative of two or more independent experiments.

isolated from AMPKα1-/- bone marrow (Fig. 16 A and B). These results suggest that in macrophages, AMPK activity is not necessary for PTEN phosphorylation or expression, but increased AMPK activity enhances the suppression of PTEN activity, thereby favoring the conversion from PIP2 to PIP3 and thus promoting the co-localization of PDK1 and Akt.

The data in Figure 15 showed that IL-10 stimulation induced Akt activation in AMPKα1+/+ macrophages, suggesting that Akt is a part of IL-10-mediated signaling pathways. The fact that inhibition of PI3K activity abrogated Akt activation indicated that IL-10 induced Akt activation depends on PI3K activity (Fig. 15). These results also suggest that PDK1 may also be a part of the IL-10-induced activation of PI3K/Akt pathway. To test if PDK1 is also regulated by AMPK activity, we generated macrophages from AMPKα1+/+ and AMPKα1-/- mice and stimulated the cells with IL-10. Although the phosphorylation of PDK1-Ser241 site is constitutively active, stimulation with IL-10 still induced a slight increase of p-PDK1-Ser241 in AMPKα1+/+ macrophages where as this effect was not observed in AMPKα1-/- macrophages (Fig. 17). Interestingly, like the total-PI3K p55 level (Fig. 14A), the overall total-PDK1 expression level was also reduced in AMPKα1-/- macrophages (Fig. 17). This result suggested that AMPK expression in macrophages has a positive effect in the maintenance of PDK1 activity and expression.

The data in Figures 14-17 have suggested a positive role of AMPK in the regulation of Akt upstream protein kinases in response to IL-10 stimulation in

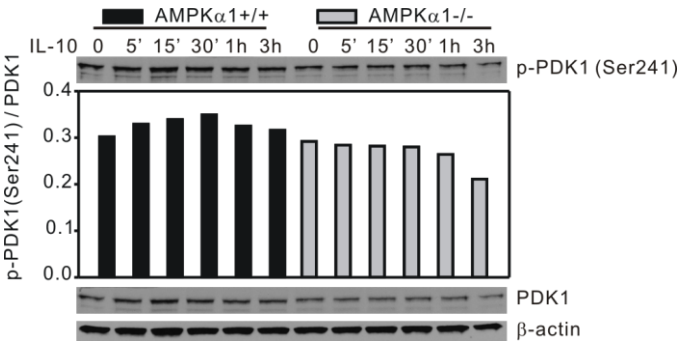

Figure 17. AMPK positively regulates PDK1 activity in response to IL-10. BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for PDK1-Ser241 phosphorylation. PDK1 activation level was analyzed by densitometry and displayed as bar histogram. The results shown are representative of two independent experiments.

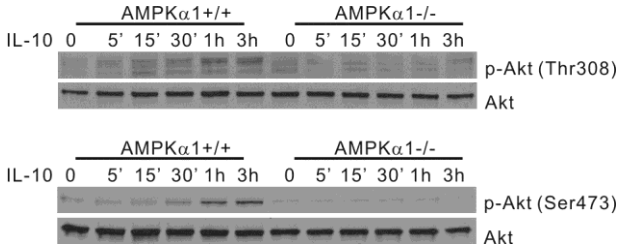

Figure 18. AMPK is required for IL-10-induced Akt activation. BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for p-Akt (Thr308) (upper panel) and p-Akt (Ser473) (lower panel) expression. The results shown are representative of more than three independent experiments.

macrophages. We then tested the influence of AMPK in Akt activation in IL-10 signal transduction. The level of Akt phosphorylation in AMPKα1-/- macrophages was greatly reduced as compared with levels present in AMPKα1+/+ macrophages. Phosphorylation of Thr308 was reduced ~3.5 fold (Fig. 18, upper panel) and phosphorylation of Ser473 was reduced ~8.5 fold (Fig. 18, lower panel) indicating a significant impairment of IL-10's ability to activate Akt in the absence of AMPKα1 expression.

Overall, results from Figure 14-18 demonstrate that IL-10 activation of AMPKα1 is upstream of PI3K/Akt signaling in macrophages.

AMPK positively regulates IL-10 activation of Akt/CREB pathway

Activated Akt regulates numerous cellular functions including cytokine expression, apoptosis, and proliferation (228, 229). The IL-10 PI3K/Akt/GSK3β pathway is known to mediate the IL-10 suppression of LPS-induced NF-κB inflammation (190, 206). We previously reported evidence that supports a positive role of AMPK in the regulation of the LPS-induced GSK3β/CREB pathway in macrophages (14). Based on these studies and the data supporting positive AMPK regulation of PI3K/Akt pathway, the role of AMPK in the regulation of IL-10-mediated GSK3β/CREB pathway was tested. The level of GSK3β phosphorylation remains intact in IL-10 stimulated macrophages in repeated experiments (Fig. 19A). Interestingly IL-10 stimulation induced robust CREB-Ser133 phosphorylation despite the unresponsiveness of GSK3β phosphorylation to IL-10. IL-10 induced CREB activation was abrogated in DN-AMPKα1 macrophages but was increased in CA-AMPKα1 macrophages (Fig. 19B).

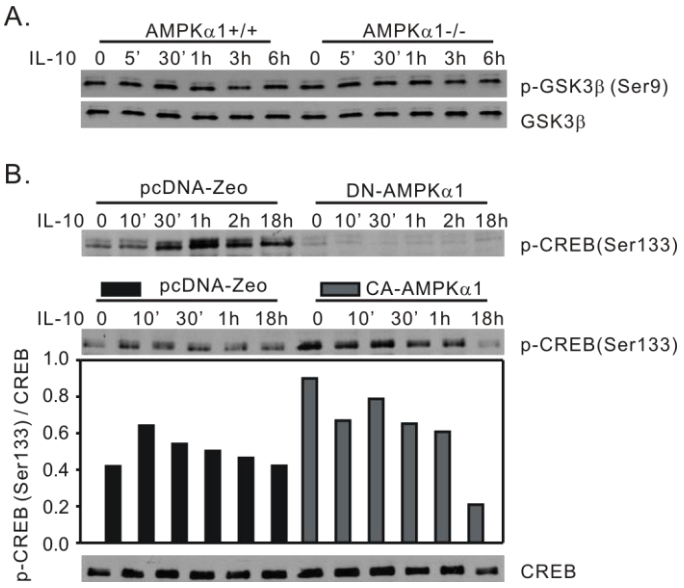

Figure 19. AMPK positively regulates IL-10 activation of GSK3β/CREB pathway. (**A**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for p-GSK3β (Ser9) and t-GSK3β expression. (**B**), CA-AMPKα1, DN-AMPKα1, and the control pcDNA-Zeo macrophage cell line were treated with rm-IL-10 (20ng/ml) for the indicated times. Cell lysates were analyzed by Western blot for CREB-Ser133 phosphorylation. CREB activation level was analyzed by densitometry and displayed as bar histogram. The results shown are representative of two or more independent experiments.

This pattern demonstrates that in response to IL-10 stimulation, activated AMPK promotes Akt activation that leads to enhancement of CREB activity.

AMPK positively regulates IL-10 activation of mTORC1/S6K pathway

Activity of the kinase complex mTORC1 is promoted by Akt phosphorylation of mTOR on Ser2448 (204, 230) and by Akt phosphorylation/inhibition of the mTORC1 inhibitory protein tuberous sclerosis complex protein 2 (TSC2) (residue Thr1462 and Ser939) (231, 232). On the other hand, AMPK restricts mTORC1 activity by phosphorylation/activation of TSC2 (residue Ser1345 and Thr1227) under conditions of energy stress in non-immune cells to limit energy consuming metabolic pathways (223). In contrast, in macrophages, transfection with a constitutively active form of CaMKIα, an AMPK upstream kinase, resulted in dramatically elevated AMPK activation accompanied by enhanced mTORC1-Ser2448 phosphorylation and treatment with AMPK inhibitor Compound C abolished this effect, indicating positive regulation of mTORC1 by AMPK (224). Emerging evidence support a negative role of mTORC1 to regulate inflammatory responses in myeloid immune cells (221, 222). Given the observed AMPK-dependent Akt activation in response to IL-10 stimulation (Fig. 18), the role of mTORC1 in this signaling pathway was evaluated. We found that IL-10 stimulus induced phosphorylation of mTOR-Ser2448 in AMPKα1+/+ macrophages with a peak of ~1.5 fold increase at 30 min post-stimulus. However, in comparison with AMPKα1+/+ macrophages, both baseline and IL-10-induced levels of phospho-mTOR-Ser2448 were reduced in AMPKα1-/- cells (Fig. 20A). In consistent, the CA-AMPKα1 macrophages, elevated AMPK activity, indicated by elevated p-ACC-Ser79 level, resulted in elevated mTOR

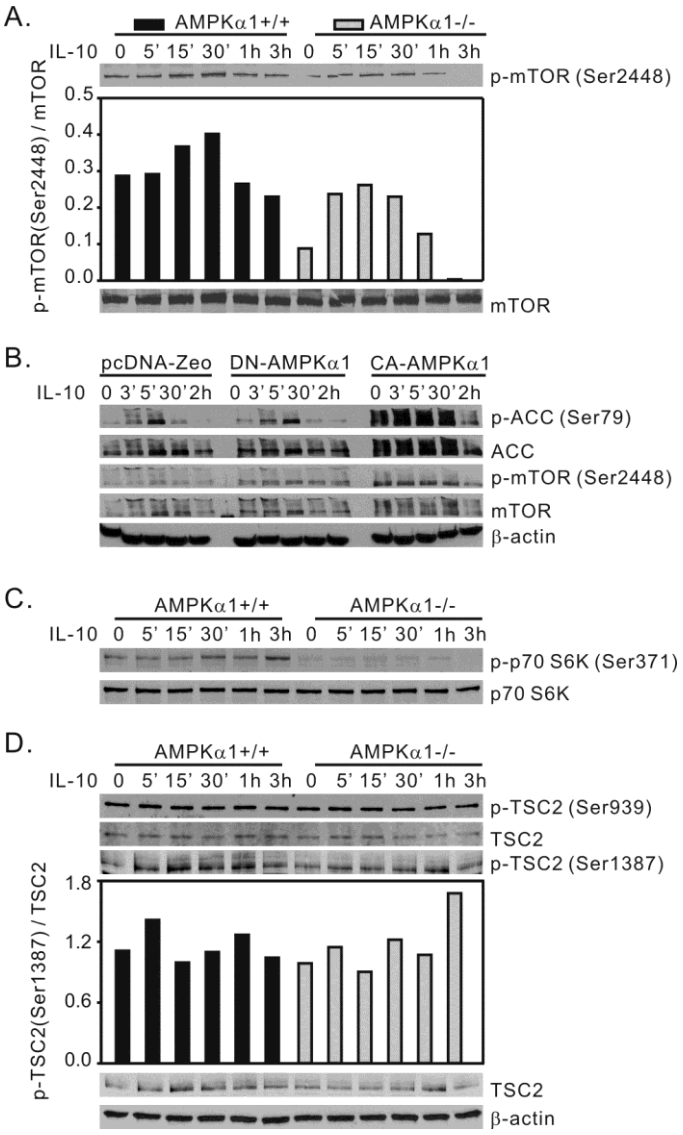

Figure 20. AMPK positively regulates IL-10 activation of mTORC1/S6K pathway. (**A**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for p-mTOR (Ser2448) and t-mTOR expression. (**B**), CA-AMPKα1, DN-AMPKα1, and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for indicated time. Cell lysates were analyzed by Western blot for p-ACC (Ser79), t-ACC, p-mTOR (Ser2448), t-mTOR, and β-actin expression. (Continued on next page…)

Figure 20, continued. (**C** and **D**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for (**C**) p-p70 S6K (Ser371) and t-p70 S6K expression, and (**D**) p-TSC2 (Ser939), p-TSC2 (Ser1387), t-TSC2, and β-actin expression. Protein phosphorylation and expression levels were analyzed by densitometry and are displayed as a bar histogram. The results shown are representative of two or more independent experiments.

phosphorylation on Ser2448 (Fig. 20B). This positive regulation of AMPK with mTORC1 activity was further demonstrated by the phosphorylation of p70 S6 Kinase (p70 S6K), a key downstream substrate of mTORC1, which, likewise, was efficiently activated by IL-10 in AMPKα1+/+, but not AMPKα1-/- macrophages (Fig. 20C). Meanwhile, there was no influence of IL-10 on the phosphorylation levels of either TSC2-Ser939 (Akt substrate) or Ser1387 (AMPK substrate) in either AMPKα1+/+ or AMPKα1-/- BMDM (Fig. 20D). These data suggest a positive role of AMPK in IL-10-induced mTORC1 activation via enhancement of PI3K/Akt activity, a regulatory effect that is TSC2-independent in macrophages.

AMPK is required for IL-10 activation of JAK/STAT3/SOCS3 pathway

The JAK/STAT3/SOCS3 signaling cascade is an important contributor to the antiinflammatory activity of IL-10 (184, 210, 211). JAK kinase activity is responsible for IL-10 induced STAT3 tyrosine phosphorylation. However, phosphorylation at both tyrosine and serine residues is necessary for STAT3 dimerization, translocation to the nucleus, and binding to the promoter regions of the *socs3* gene to initiate SOCS3 protein expression. SOCS3 is a rapidly produced and quickly degraded protein with a potent ability to suppress TLR-mediated inflammation (185, 219, 220). In our evaluation of the role of AMPK in IL-10-induced JAK/STAT3/SOCS3 pathway, ~4.5 fold increase of JAK1 phosphorylation was observed in AMPKα1+/+ BMDM, an effect that was greatly reduced in AMPKα1-/- BMDM (Fig. 21A). To further address the relationship between IL-10 induced AMPK and JAK kinase activity, we pretreated BMDM generated from wild-type C57BL/6J mice with a variety of JAK kinase inhibitors (the JAK2 inhibitor

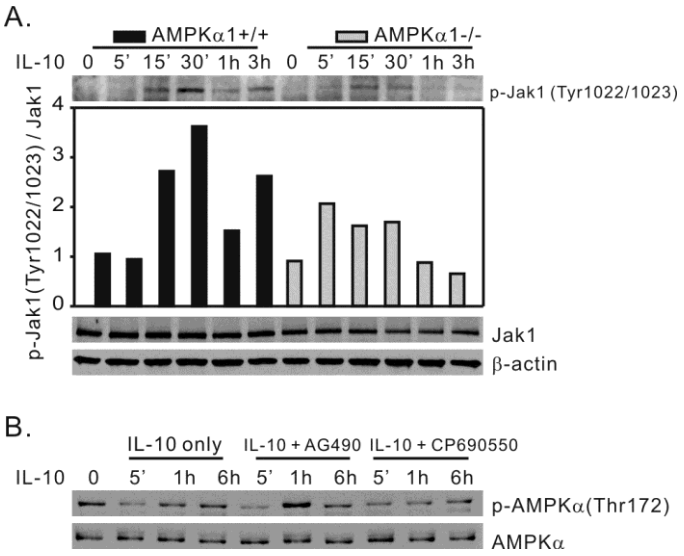

Figure 21. AMPK positively regulates JAKs activity in response to IL-10. (**A**), BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for p-JAK1 (Tyr1022/1023) and t-JAK1 expression. JAK1 phosphorylation and expression levels were analyzed by densitometry and are displayed as a bar histogram. (**B**), BMDM generated from C57BL/6 mice were incubated with either media alone, or with JAK2 inhibitor AG490 (25 μM), or JAK3 inhibitor CP690, 550 (25 nM) for 1 h, then exposed to rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for AMPK-Thr172 phosphorylation. The results shown are representative of two to four independent experiments.

AG490 and the JAK 3 inhibitor CP-690, 550), then stimulated the cells with IL-10. Cell lysates were collected at the time points shown in Fig. 21B for Western blot analysis of phospho-AMPK levels. The efficacy of the JAK kinase inhibitors was demonstrated by impaired JAK phosphorylation in response to IL-10 stimulation (data not shown). As shown in Fig. 21B, AMPK activation by IL-10 stimulation was not affected by pre-treatment with these inhibitors.

IL-10-induced JAK1 activation is accompanied with elevated phosphorylation of both STAT3 Tyr705 (Fig. 22A, upper panel) and Ser727 (Fig. 22A, lower panel) in AMPKα1+/+ BMDM, whereas AMPKα1-deficiency completely abrogated this influence of IL-10. The phosphorylation of Tyr705 occurred within 5 min whereas the phosphorylation of Ser727 occurred with slightly delayed onset, but both phosphorylation events peaked at 30 min - 1h (Fig. 22A). This AMPK-dependent STAT3 activation by IL-10 is further confirmed by the utilization of the pharmaceutical inhibitor STO-609 to block AMPK activity. STO-609 at concentrations between 1-10 μg/ml (2.6 - 26 μM) effectively blocks AMPK signaling cascades via inhibition of its upstream regulatory kinase CaMKKβ (233). BMDM derived from AMPKα1+/+ mice were pre-incubated with STO-609 (5 μM) for 1h prior to stimulation with IL-10. Cell lysates were harvested at the indicated time points and were analyzed by Western blot for AMPK and STAT3 phosphorylation. Pre-incubation with STO-609 blocked IL-10 induced AMPKα-Thr172 phosphorylation (Fig. 22B, upper panel) and inhibited IL-10-induced STAT3 phosphorylation of both Tyr705 (Fig. 22B, upper panel) and Ser727 (Fig. 22B, lower panel). The observation that IL-10 induced higher STAT3-Tyr705 phosphorylation in

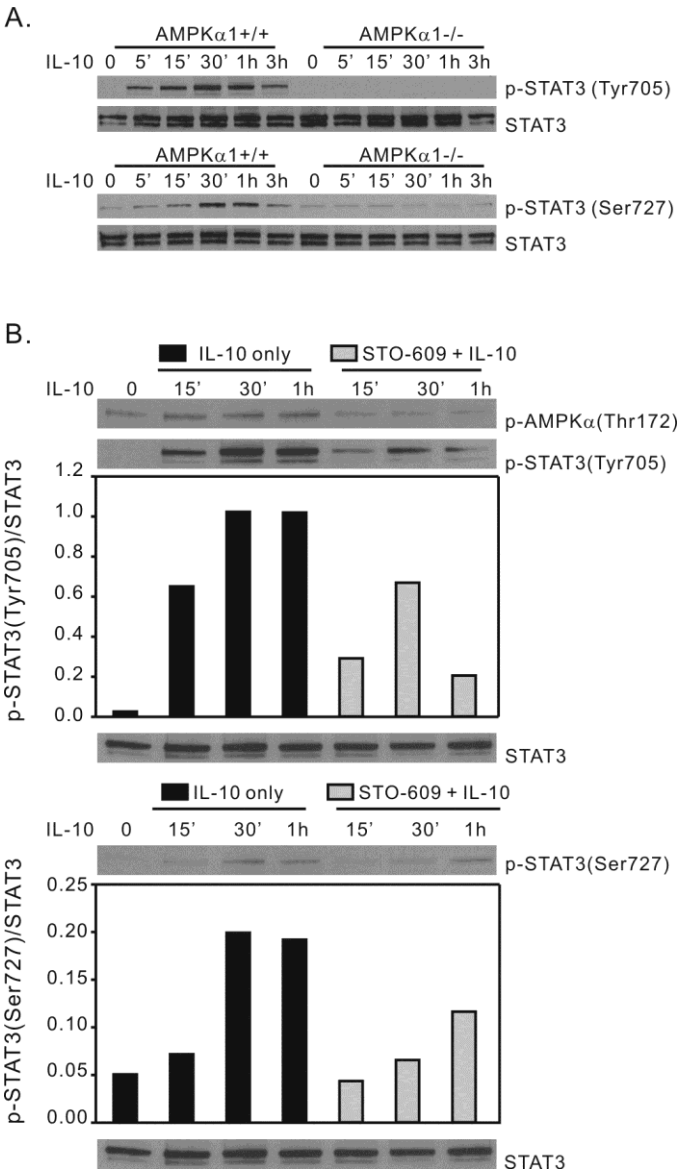

Figure 22. IL-10-induced STAT3/SOCS3 activation requires AMPK activity. BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for (**A**), STAT3-Tyr705 (upper panel) and STAT3-Ser727 (lower panel) phosphorylation, and (**D,** upper panel), SOCS3 and β-actin expression. (Continued on next page…)

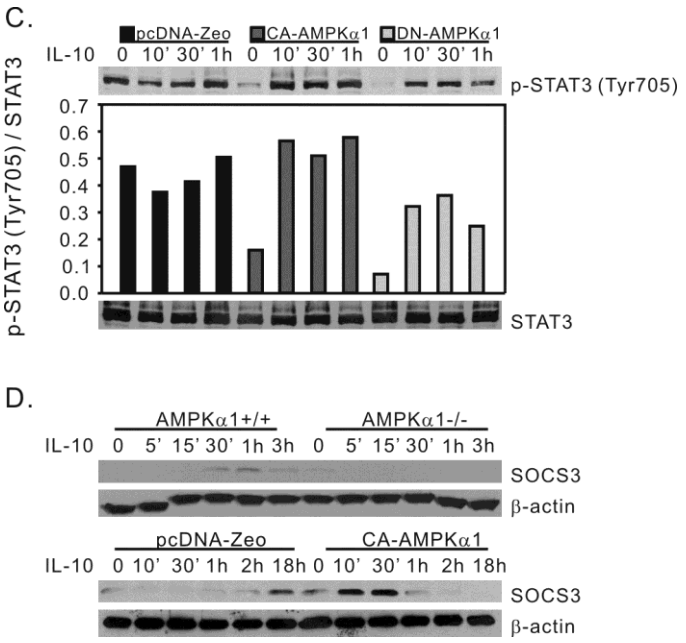

Figure 22, continued. (**B**), BMDM generated from C57BL/6 mice were incubated with either media alone, or with CaMKKβ inhibitor STO-609 (5 μM) for 1 h, then exposed to rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for AMPK-Thr172, STAT3-Tyr705 (upper panel) and STAT3-Ser727 (lower panel) phosphorylation. (**C**), CA-AMPKα1, DN-AMPKα1, and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for indicated time. Cell lysates were analyzed by Western blot for STAT3-Tyr705 phosphorylation. STAT3 activation levels were analyzed by densitometry and are displayed as a bar histogram. (**D,** lower panel), CA-AMPKα1 and the control macrophage cell line were treated with rm-IL-10 (20ng/ml) for indicated time. Cell lysates were analyzed by Western blot for SOCS3 and β-actin expression. The results shown are representative of two or more independent experiments.

CA-AMPKα1 macrophages also supported a positive role of AMPK in promoting IL-10 activation of STAT3 (Fig. 22C). In our analysis of SOCS3 mRNA in response to IL-10, an example of which is shown in Fig. 8, we observed SOCS3 mRNA accumulation in less than 30 min in AMPKα1+/+ cells (data not shown). Consistent with this result, IL-10 treatment induced the rapid appearance of SOCS3 protein in AMPKα1+/+ cells, which peaked at 30 min - 1h, yet we were unable to detect SOCS3 protein in IL-10-stimulated AMPKα1-/- BMDM (Fig. 22D upper panel). Measurement of SOCS3 in CA-AMPKα1 macrophages revealed a more stable baseline expression (Fig. 22D lower panel), providing the supportive evidence of AMPK positive regulation of SOCS3 stable expression.

Data in Figures 21 and 22 suggested an essential role of AMPK in promoting IL-10-induced activation of JAK/STAT3 pathway that leads to expression of SOCS3 protein.

mTORC1 activity is required for optimal STAT3 activation

The AMPK-dependency of both tyrosine and serine phosphorylation of STAT3 suggested the possible convergence of AMPK-directed tyrosine kinase (JAK1) activities and serine kinase (mTORC1) activities in IL-10-directed STAT3 activation. Data in Figures 21 and 22 provided evidence of AMPK-directed Jak1 kinase activity. The mTORC1 inhibitor rapamycin was used to determine a possible role of mTORC1 in the IL-10-mediated serine phosphorylation of STAT3. Pretreatment of BMDM with rapamycin suppressed IL-10-induced S6K phosphorylation (Fig. 23A, upper panel), without influencing AMPK activity (Fig. 23A, lower panel). Rapamycin pre-treatment

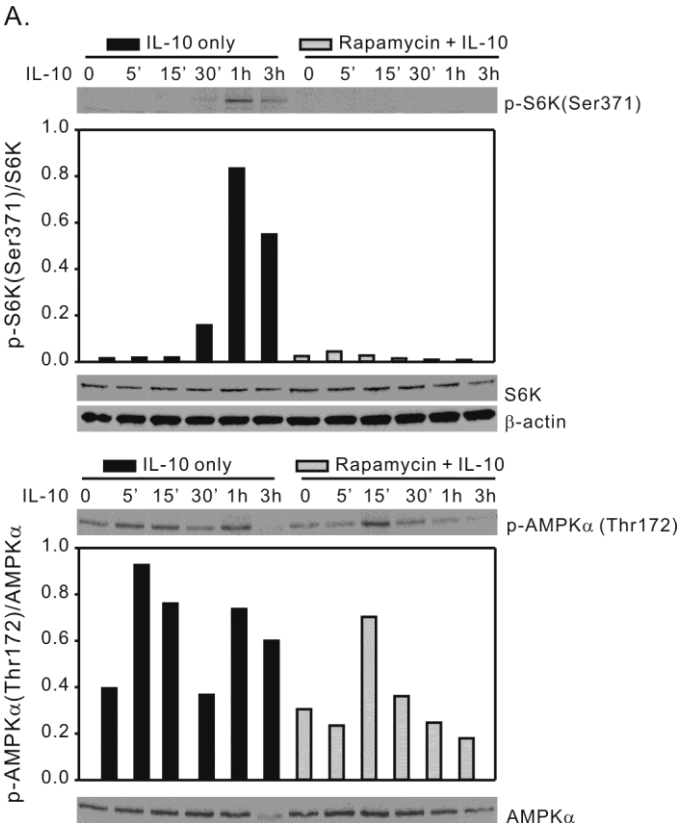

Figure 23. Inhibition of mTORC1 activity leads to impaired STAT3 phosphorylation. BMDM generated from C57BL/6 mice were incubated with either media alone, or with the mTORC1 inhibitor rapamycin (100 ng/ml) for 1 h and then exposed to rm-IL-10 (20 ng/ml) for the time points indicated. Cell lysates were analyzed by Western blot for (**A**) p70 S6K-Ser371 (upper panel) and AMPK-Thr172 (lower panel) phosphorylation and (**B**) STAT3-Ser727 (upper panel) and STAT3-Tyr705 (lower panel) phosphorylation.

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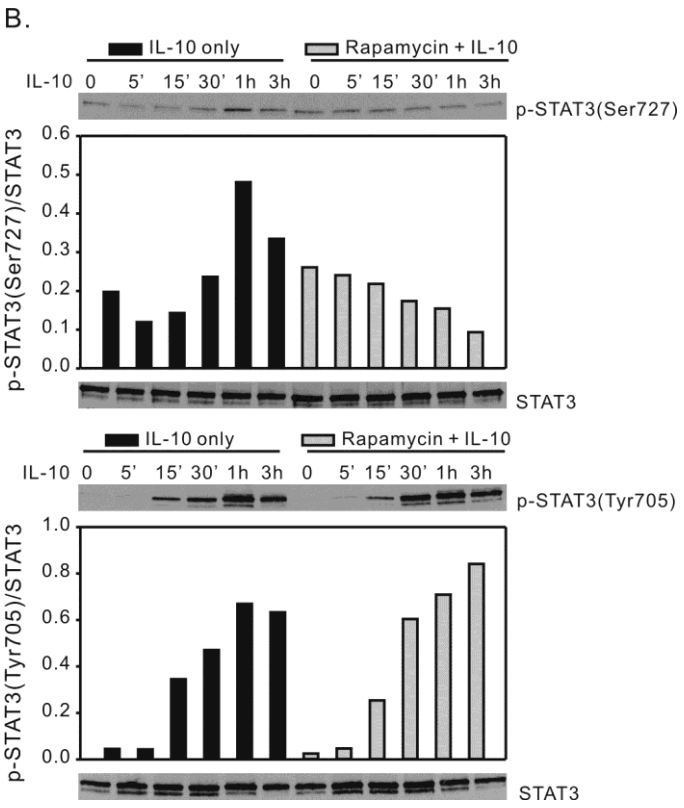

Figure 23, continued. Protein phosphorylation levels were analyzed by densitometry and are displayed as a bar histogram. Results shown are representative of three independent experiments.

resulted in blockade of STAT3 Ser727 phosphorylation (Fig. 23B, upper panel), without effect on Tyr705 phosphorylation (Fig. 23B, lower panel).

The results in Figures 20-23 indicate an upstream role of AMPK in the activation of both the tyrosine kinase (JAK1) and serine kinase (mTORC1) activities necessary for optimal activation of STAT3 via IL-10 stimulation in macrophages.

AMPK contributes to IL-10 suppression of LPS-induced proinflammatory cytokine production

The STAT3/SOCS3 pathway has been shown to play a major role in IL-10 suppression of LPS-induced TLR inflammation. For example, overexpression of a constitutively active form of STAT3 mimicked IL-10 suppression of LPS-induced TNFα and IL-6 production in primary human macrophages (234). In contrast, deficiency of STAT3 expression in murine macrophages resulted in an inability of IL-10 to inhibit LPS-induced TNFα production (214). In addition, it has been reported that macrophages derived from mice with myeloid-specific STAT3-deletion produce significantly increased TNFα, IL-6, and IL-12 in response to LPS stimulation, and the ability of IL-10 to limit LPS-induced TNFα and IL-6 production is greatly reduced in these macrophages (235). SOCS3 was found to be responsible for IL-10 inhibition of LPS-induced TNFα and NO production in macrophages (220). Our data indicating AMPK's role in induction of STAT3 activation and SOCS3 expression suggested that AMPK activity should impact IL-10's suppressive influence on TLR responses in macrophages. As shown in Fig. 6, stimulation with LPS (10 ng/ml) induced expression of the proinflammatory cytokines

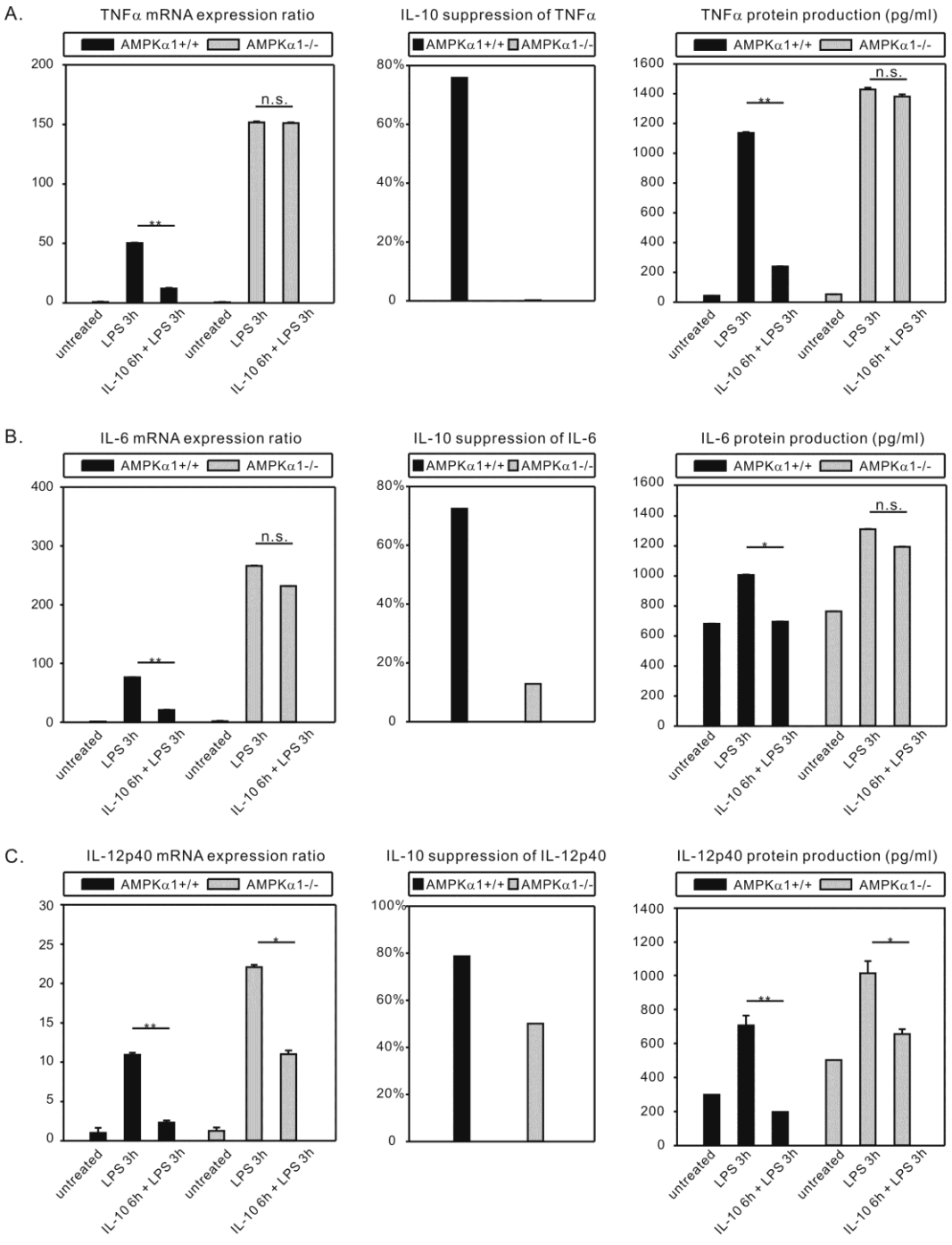

Figure 24. AMPK contributes to IL-10-mediated suppression of LPS-induced TNFα (**A**), IL-6 (**B**) and IL-12p40 production (**C**). BMDM generated from AMPKα1+/+ and AMPKα1-/- mice were treated with rm-IL-10 (20 ng/ml) for 6h, then exposed to LPS (10 ng/ml) for 3 h. (Continued on next page…)

Figure 24, continued. Total cellular lysates were collected for real-time PCR analysis (left panels). The ability of IL-10 to suppress LPS-mediated responses, based on the RT-PCR data, is depicted as % suppression (middle panels). Supernatants were collected for analysis by ELISA (right panels). RT-PCR data shown are mean ± SD of triplicate determinations. ELISA data shown are mean ± SEM of triplicate determinations. Statistical significance between groups was calculated with an unpaired Student's *t* test, with a value of $p < 0.050$ considered statistically significant. (**, $p < 0.001$. *, $p < 0.050$. n.s., $p > 0.050$). The data shown are representative of two or three independent experiments.

TNFα, IL-6, and IL-12p40 in both AMPKα1+/+ and AMPKα1-/- BMDM at the mRNA and protein (ELISA) levels. As we have shown previously, AMPKα1-/- BMDM produced higher levels of TNFα and IL-6 (14, 15) and, in addition, higher levels of IL-12p40 (Fig. 24). Pre-treatment with IL-10 resulted in strong suppression (~80%) of LPS-induced TNFα, IL-6, and IL-12p40 mRNA transcription (Fig. 24). However, in AMPKα1-/- BMDM, IL-10 suppression of LPS-induced expression of TNFα and IL-6 was nearly absent, and suppression of IL-12p40 expression was substantially reduced as compared to that observed in AMPKα1+/+ BMDM (Fig. 24).

Overall, our data suggest an essential role of AMPK in regulating IL-10 suppressive signaling pathways that promotes immune suppressive functions of macrophages induced by IL-10 (Fig. 25). AMPK is quickly activated by IL-10 stimulation in macrophages. IL-10 signaling promotes the rapid phosphorylation of JAK1 in an AMPK-dependent manner. Activation of JAK1 subsequently leads to the phosphorylation and activation of STAT3 (Tyr 705), which is critical for SOCS3 production. In addition to its role in JAK/STAT signaling, AMPK also simultaneously promotes the activation of PI3K by enhancing the phosphorylation of its p55 subunit. Phospho-activation of Akt rapidly follows, leading to an increase in mTORC1 activity as reflected by an increase in S6K phosphorylation (Ser371). Activation of mTORC1 leads to an increase in phosphorylation of STAT3 (Ser727), which further enhances STAT3 transcriptional activity leading to SOCS3 production. Expression of SOCS3 in turn suppresses TLR-activated inflammatory cytokines production. Additionally, AMPK positively contributes to IL-10 induced Akt

downstream CREB activation that is responsible for IL-10 production that enhances macrophage antiinflammatory functions.

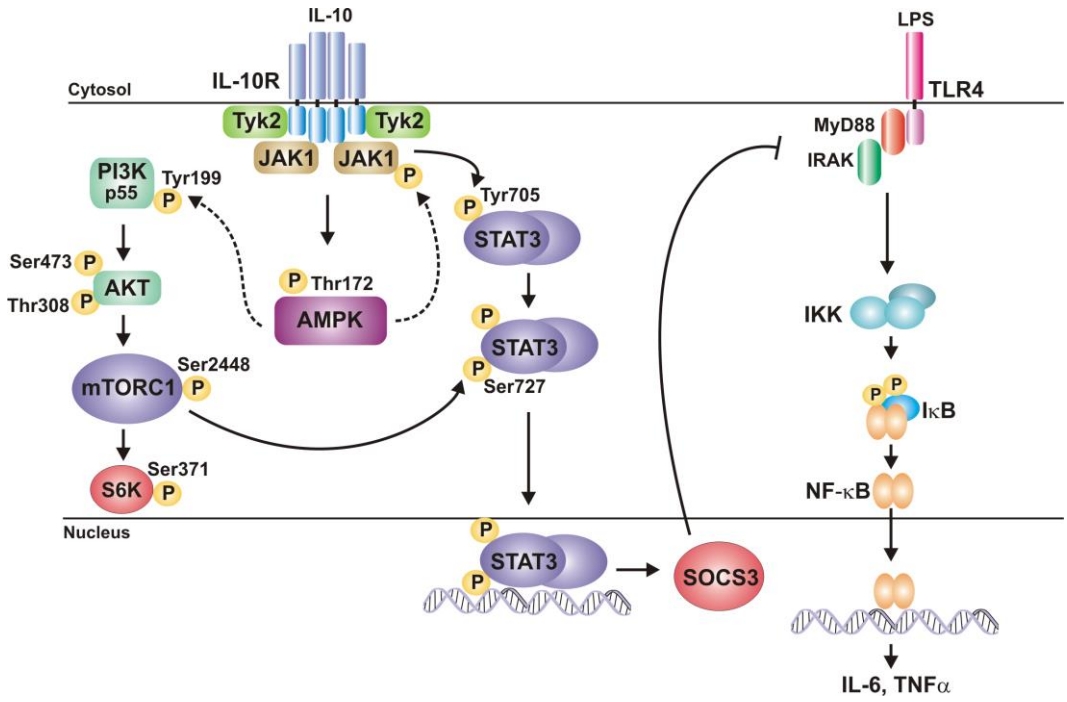

Figure 25. AMPK activation promotes the antiinflammatory properties of IL-10 through bifurcated activation of the Akt/mTORC1 and JAK/STAT signaling pathways. IL-10 signaling promotes the rapid phosphorylation of JAK1 in an AMPK-dependent manner. Activation of JAK1 subsequently leads to the phosphorylation and activation of STAT3 (Tyr 705), which is critical for SOCS3 production. In addition to its role in JAK/STAT signaling, AMPK also simultaneously promotes the activation of PI3K by enhancing the phosphorylation of the p55 subunit. Phospho-activation of Akt rapidly follows, leading to an increase in mTORC1 activity, reflected by an increase in S6K phosphorylation (Ser371). Activation of mTORC1 leads to an increase in phosphorylation of STAT3 (Ser727), which further enhances STAT3 transcriptional activity leading to SOCS3 production. Expression of SOCS3 in turn suppresses TLR-activated inflammatory cytokines production.

DISCUSSION

Macrophages display profound phenotypic and functional heterogeneity due to their ability to adapt to the tissue microenvironment (1). This functional plasticity is crucial to their response to tissue damage, clearance of pathogens, contribution to adaptive immune responses, and wound resolution. Macrophages can respond rapidly and reversibly to cytokine stimuli and in doing so can change functional phenotype (7). The ability of macrophages to be polarized to inflammatory and antiinflammatory states, e.g., via treatment with LPS + IFNγ or IL-4, respectively, has been studied extensively for over a decade, including attempts to elucidate the intracellular signaling pathways controlling the polarization process (236, 237). We identified AMPK as a negative regulator of LPS-induced inflammatory responses in macrophages and dendritic cells via induction of a PI3K/Akt/GSK3β/CREB pathway and inhibition of NF-κB activity (14, 15). Likewise, Yang *et al.,* reported that AMPK activity inhibited fatty acid-induced inflammation in macrophages in a SIRT1-dependent manner (109). In the current report we follow-up on the finding that antiinflammatory cytokines such as IL-10, IL-4 and TGFβ are efficient activators of AMPK (14) and examined the role of AMPK in IL-10 signal transduction and antiinflammatory function.

IL-10 induces expression of a number of genes encoding proteins with antiinflammatory and atheroprotective function. We began by asking the simple question

of whether or not AMPKα1 contributes to IL-10-induced gene expression. Our data provide direct evidence that IL-10 induces mRNA transcription of important atheroprotective genes in macrophages including apoE, LXRα, and ABCA1, as well as the immune suppressive SOCS family proteins in an AMPK-dependent manner (Fig. 8). The influence of AMPKα1 on apoE expression is supported by the matching data that macrophages transfected with constitutively active-AMPKα1 expressed ~ 60 fold higher apoE mRNA as compared to empty-vector transfected macrophages, while macrophages transfected with AMPKα1 siRNA did not express apoE in response to IL-10 (Fig. 9A and C). Interestingly, as is the case for AMPK (14), ApoE has been recognized as a regulator of macrophage plasticity that promotes an antiinflammatory "M2-like" phenotype (182, 183).

SOCS3 is rapidly induced by IL-10 and potently inhibits inflammatory responses in many cell types including macrophages (185, 186). Our data demonstrate a requirement for AMPKα1 expression for IL-10 induced SOCS3 expression at both the mRNA (Fig. 8) and protein (Fig. 22D upper panel) level. This positive regulatory role of AMPKα1 in SOCS3 expression is also supported by the elevated SOCS3 protein expression in constitutively active-AMPKα1 transfected macrophages as compared to empty-vector transfected macrophages (Fig. 22D lower panel). In our investigation of the mechanisms underlying AMPK's role in IL-10-mediated SOCS3 expression we found that IL-10 induced rapid PI3K activation at 5-15 min (Fig. 14) and phosphorylation of both Akt Thr308 and Akt Ser473 (Fig. 18). The Akt downstream target mTORC1 was likewise activated as indicated by both mTOR phosphorylation and phosphorylation of the mTORC1 target p70 S6K (Fig. 20). Each of these IL-10-induced phosphorylation

events were reduced in AMPKα1-/- BMDM as compared to BMDM derived from AMPKα1+/+ mice (Figs. 18 and 20). Neither the PI3K inhibitor LY294002 nor the mTORC1 inhibitor rapamycin affected IL-10 induced AMPK activity (Fig. 15 and Fig. 23), indicating an upstream regulatory role of AMPK IL-10 activation of the PI3K/Akt/mTOR pathway.

Phosphorylation of Akt at Thr308 is mediated by PDK1, downstream of PI3K. Thus, the upstream role of AMPK can explain its impact on phosphorylation of Akt on this site. The Rictor-mTOR complex (mTORC2) has been suggested to mediate phosphorylation of Akt Ser473 (238, 239). However, the regulatory mechanism of mTORC2 activation is still poorly understood. The demonstration that IL-10 induces phosphorylation of Akt at Ser473 in an AMPK-dependent manner suggests that AMPK influences mTORC2 activity in macrophages via an as yet undescribed mechanism.

Akt is a central mediator of many signaling cascades involved in different biological processes. PI3K/Akt/GSK3β pathway is one of those cascades that take responsibility to IL-10 suppression of LPS-induced proinflammatory gene expression via the inhibition of NF-κB activation (190, 206). Our previous research demonstrated that CA-AMPKα1 macrophages display elevated phosphorylation/inactivation of GSK3β accompanied with increased CREB phosphorylation/activation in response to LPS stimulation (14). We therefore investigated if IL-10 signaling also initiates the activation of this pathway. Phosphorylation level of GSK3β stayed intact in both AMPKα1+/+ and AMPKα1-/- BMDM in response to IL-10 stimulation (Fig. 19A), whereas IL-10 stimulation induced diminished CREB activation in DN-AMPKα1 compared to control macrophages while CREB activation was elevated in CA-AMPKα1 (Fig. 19B). Both

mTORC1 (221) and CREB (240) are suggested to be responsible for IL-10 gene expression. Although other mechanisms may provide feedback compensation effects on GSK3β activity based on the unresponsiveness of GSK3β phosphorylation by IL-10 stimulation (Fig. 19A), the positive regulation between AMPK and CREB activation (Fig. 19B) and between AMPK and mTORC1 (Fig. 20) both suggested that AMPK is important for IL-10 activation of PI3K/Akt downstream pathways that lead to antiinflammatory responses such as IL-10 production in macrophages.

Studies on non-immune cells have shown that mTORC1 activity is negatively regulated via phosphorylation/activation of TSC2 on by AMPK (residue Ser1387 and Thr1227) under conditions of energy stress, and is positively regulated via phosphorylation/inactivation of TSC2 (Ser929) by Akt in response to insulin stimulation (223, 231). Additionally, Akt positively regulates mTORC1 activity via direct phosphorylation of mTOR-Ser2448 residue (204). In our study we observed decreased mTOR-Ser2448 in AMPKα1-/- BMDM but did not observe changes in phosphorylation of either TSC2 Ser1387 or TSC2 Ser939 levels in AMPKα1+/+ or AMPKα1-/- BMDM in response to IL-10 (Fig. 20D). These data suggest that the AMPK's influence on mTORC1 in macrophages in response to IL-10 stimulation is Akt-dependent, but independent of TSC2.

Activation of the PI3K/Akt/mTORC1 pathway in macrophages is shown to elicit an antiinflammatory phenotype including induction of IL-10 expression (190, 191, 221, 222). Activated mTORC1 can also influence STAT3 activity via phosphorylation of STAT3 on Ser727. We found diminished STAT3 phosphorylation on both Tyr705 and Ser727 sites in AMPKα1-/- macrophages as well as AMPKα1+/+ macrophages treated

with an inhibitor of CaMKKβ, an upstream activator of AMPK (Figs. 22). The data suggest that STAT3 in response to IL-10 is a two-step mechanism involving AMPK dependent regulation of both JAK kinase and mTORC1-mediated STAT3 phosphorylation. This conclusion is supported by our observation that both JAK1 and mTOR phosphorylation were impaired in AMPKα1-/- BMDM in response to IL-10 stimulation (Figs. 21A and 20A), and that pretreatment with JAKs inhibitors (AG490 and CP-690, 550) or mTORC1 inhibitor (rapamycin) did not affect IL-10 induced AMPK and PI3K activation (Figs. 21B, 23A, and data not shown). The impaired STAT3 activity in AMPKα1-/- BMDM is accompanied by diminished SOCS3 protein production (Fig. 22D), a result that is consistent with the reduced SOCS3 mRNA level observed in AMPKα1-/- BMDM (Fig. 8).

The lack of SOCS3 expression in response to IL-10 stimulation in AMPKα1-/- BMDM suggested that the suppressive function typically induced by IL-10 would likely by impaired. Indeed, our data revealed a notable contribution of AMPKα1 towards the ability of IL-10 to suppress LPS-induced macrophage proinflammatory cytokine production (Fig. 24). In addition to activation of the STAT3/SOCS3 pathway, a number of other mechanisms have been shown to mediate IL-10's suppressive function. For example, prolonged IL-10 pre-incubation is able to suppress LPS-induced TNFα production in the absence of SOCS3 expression in macrophages (220). Proposed mechanisms for IL-10 suppression of LPS-induced IL-12p40 production include reduced RNA polymerase II recruitment to the p40 promoter (241) and promoter histone deacetylation (242). A role for both STAT3/SOCS3 dependent and independent mechanism of IL-10-medicated suppression could account for the partial effect of

AMPKα1-deficiency on IL-10 suppression of LPS-induced IL-12p40 expression (Fig. 24C).

Thus far, our evaluation of myeloid-expressed AMPKα1 reveals its function as a counter-regulator of inflammatory signaling pathways induced, for example, via TLR and CD40 stimulation (14, 15) and as a mediator of the suppressive function of the antiinflammatory cytokine, IL-10. Given the ability of numerous antiinflammatory mediators to rapidly activate AMPK in myeloid cells (14), it is likely that AMPK is a common upstream component of multiple signaling pathways, independent of metabolic stress. Although IL-10 activation of AMPK may impact downstream metabolic pathways, the AMPK-dependent antiinflammatory function induced by IL-10 appears to be independent of this effect. IL-10 is well-established as a critical mediator of immune homeostasis, in mice and humans, with the ability to dampen the destructive effects of inflammation in numerous pathologies (160). Thus, the identification of AMPK as a mediator of IL-10 action supports continued exploration of therapies directed towards the modulation of AMPK action.

CHAPTER 4

CONCLUSIONS AND FUTURE PERSPECTIVES

Macrophages are extremely versatile immune cells with the remarkable ability to rapidly and reversibly respond to cytokine stimuli and adaptively change their functional phenotype (7). This functional plasticity is critically correlated to atherosclerosis disease progression and recession (127, 128). The physiological microenvironment within atherosclerosis lesions contains diverse factors with opposite roles in inflammation. For example, IFNγ and IL-10, have the potential to promote macrophage functions that are either atherogenic or antiatherogenic, repectively. Attempts to elucidate the intracellular signaling pathways controlling macrophage polarization process have been studied extensively for over a decade (236, 237).

Herein, we present data that supports an essential role of AMPK in mediating IL-10 triggered signaling events that lead to acquisition of antiinflammatory functions of macrophages as well as expression of atheroprotective genes such as apoE, LXRα, ABCA1, PPARγ, *etc.* (Figs. 8, 9 and 12) and inhibition of LPS-induced proinflammatory cytokine productions such as TNFα, IL-6, and IL-12p40 (Fig. 24). Macrophage-specific expression of DN-AMPKα1 in transgenic mice resulted in spontaneous obesity associated with fatty liver, heart enlargement, and increased proinflammatory cytokine production in macrophages (Fig. 5, 6, and T. 1). Although the spontaneous obese

phenotype of DN-AMPKα1 mice was lost in their F2 offspring after an animal maintenance facility change, possibly due to the variation in the composition of residential bacteria (243), the critical role of macrophage expressed AMPK in the regulation of atherosclerosis was further supported by data showing decreased expression of the inflammatory cytokine IL-6 (Fig. 6A) but increased expression of the atheroprotective proteins ABCA1 (Fig. 7B) and apoE (Fig. 9A) in the CA-AMPKα1 macrophages. These data suggested an important role of macrophage-expressed AMPK in the regulation of whole body metabolic and inflammatory homeostasis. Additionally, a co-dependent relationship was discovered between AMPK and the atheroprotective transcriptional factor PPARγ (Figs. 10 and 12). In this co-dependent relationship, PPARγ-mediated IL-10 production was suggested to be responsible for PPARγ agonist-induced AMPK activation at time points > 3h after treatment (Fig. 11). These results provide plausible evidence for an important regulatory role of AMPK in macrophage antiatherogenic functions and contribute to the search for the therapeutic targets for atherosclerosis.

Further investigation demonstrated a vital role of AMPK in mediating signaling pathways that are responsible for IL-10 immune regulatory functions. These AMPKα1-regulated pathways include IL-10 activation of PI3K/Akt/CREB, PI3K/Akt/mTORC1, and JAK/STAT/SOCS3 pathways.

Our data showed rapid AMPK activation by IL-10 stimulation in macrophages (Fig. 13), which was found essential for IL-10 activation of PI3K (Fig.14), and then proved to be upstream of PI3K activation (Fig. 15). In addition to the PI3K activity

implied to be stimulated by IL-10 in pro-myeloid cells (191), our data showed for the first time that elevated phosphorylation/activation of PI3K by IL-10 in macrophage occurs through an AMPK-dependent mechanism. The new insight provides important information for the understanding of IL-10 signaling pathways.

Supportive data of AMPK regulation in IL-10 induced PI3K/Akt pathway include that constitutively active AMPK activity in macrophages resulted in decreased PTEN phosphorylation on the residues that are important to maintain PTEN stability, which may be responsible for the reduced PTEN levels (202) in these macrophages (Fig. 16A). Deficient expression of AMPKα1 in macrophages led to impaired overall expression and slightly reduced phosphorylation of PDK1 (Fig. 17), a kinase that phosphorylates Akt-Thr308 and promotes Akt activation. In agreement with these data, the result shown in Figure 18 clearly demonstrated a requirement for AMPKα1 in IL-10 activation of Akt on both threonine and serine residues, both of which contribute to optimal activation of Akt. Thus, an essential role of AMPK in the regulation of IL-10 activation of PI3K/Akt signaling pathway is demonstrated for the first time in macrophages.

Further investigation of this pathway included examination of two downstream targets of Akt, CREB (Fig. 19) and mTORC1 (Fig. 20). A mechanism for AMPK's enhancement of CREB activity was demonstrated in CA-AMPKα1 macrophage cell line stimulated with LPS (14), however the underling mechanism between LPS and IL-10 crosstalk is not fully understood. Our data suggested a positive role of AMPK activity to enhance IL-10-induced activation of CREB (Fig.19), therefore providing a possible mechanism to understand the crosstalk between IL-10 and LPS. It has been well established in non-immune cells that under metabolic stress, AMPK negatively regulates

mTORC1 activity via phosphorylation on TSC2-Ser1387 (223), however our data suggested a TSC2-independent but Akt-dependent mechanism by which AMPK enhances mTORC1 activity in response to IL-10 stimulation in macrophages (Fig. 20). This conclusion was confirmed by the fact that inhibition of mTORC1 activity by the inhibitor rapamycin failed to suppress IL-10 induced AMPK activation (Fig. 23A), and is consistent with the observation by Guo, *et al.* that AMPK activity is positively associated with mTORC1 activity in macrophages (224). This groundbreaking discovery suggests a need to reconsider carefully the use of AMPK activators such as metformin in the treatment of inflammation-associated diseases such as in atherosclerosis or cancer, as these drugs may influence inflammatory signaling pathways in immune cells totally separate from the effects they have on the metabolic pathways in non-immune cells including SMCs or tumor cells. For example, although metformin may reduce tumor growth, it may also suppress anti-tumor immunity by boosting IL-10's immunosuppressive functions.

Another important discovery is that our data suggested a requirement for both JAK-mediated tyrosine phosphorylation and mTORC1-mediated serine phosphorylation of STAT3, both of which are regulated by AMPK. Activation of the JAK/STAT3 pathway by IL-10 is well established, however, a requirement for a second signal in addition to JAK tyrosine phosphorylation to mediate IL-10-induced STAT3 function was suggested (214) but never elucidated in the literature. It has also been suggested that mTORC1-mediated serine-727 phosphorylation is important for STAT3 optimal activation (215-218), however the significance of this phosphorylation event in IL-10 signaling was never reported. By showing a requirement for both AMPK (Fig. 21 and

22) and mTORC1 (Fig.23), we are able to identify the critical regulatory role of AMPK in mediating optimal STAT3 activation via both tyrosine and serine phosphorylation in response to IL-10 stimulation in macrophages. This conclusion provided an answer to the unresolved search for a secondary signal in mediating IL-10 activation of JAK1/STAT3 pathway, and contributes to the understanding of the IL-10 signaling puzzle.

Finally, the IL-10-induced signaling cascades mediated by AMPK in macrophages led to the production of SOCS3 (Figs. 8 and 23D), which is recognized as a potent mediator of IL-10 suppressive functions. Impaired SOCS3 production due to the lack of AMPKα1 expression contributes to the inability of AMPKα1-deficient macrophages to efficiently suppress LPS-induced proinflammatory cytokine production (Fig. 24), confirming the key role of AMPK in mediating IL-10 suppressive functions in macrophages.

Thus far, the research in this dissertation provides a body of evidence to demonstrate an IL-10 initiated signaling network regulated at several critical points by AMPK (Fig. 25), which mediate macrophage antiinflammatory functions. The findings have broad-reaching implications for our understanding the mechanism of IL-10 action in the maintenance of the immune homeostasis, as well as in inflammation-associated diseases where IL-10 plays a key role in limiting excessive inflammatory responses, such as in atherosclerosis (166), bowel disease (188), EAE (189), and cancer (244, 245). The mechanisms discovered in this dissertation also contribute to our understanding of the possible outcomes of using AMPK activators as a treatment of these diseases. For

example, treatment with the AMPK activator AICAR led to protective effects in EAE (102) and colitis (103). Another AMPK activator, metformin, has been used to treat T2D and atherosclerosis (69, 70) and recently the use of metformin has been suggested to have beneficial effects in reducing tumor growth (246). However the mechanisms of these drugs are not fully understood.

Therefore, this study in the understanding of AMPK as a key regulator of IL-10 suppressive pathways in macrophages has great values in answering the question of what is the underlying mechanism of both macrophage polarization and IL-10 antiinflammatory functions that has been investigated over decades (210, 236, 237), and also has provided additional insights into the use of AMPK as a therapeutic target to treat inflammation-associated diseases such as atherosclerosis, autoimmune diseases and cancer.

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CURRICULUM VITAE

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EDUCATION

2008-present	Ph.D. Candidate, Microbiology and Immunology, Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY USA
2008-2011	M.S., Microbiology and Immunology, Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, KY USA
2003-2007	B.Sc., Biotechnology, Life Science College, Northwest A & F University, China

RESEARCH AND TRAINING EXPERIENCE

Position: Graduate Research Assistant
Mentor: Jill Suttles, Ph.D., Professor
Department of Microbiology and Immunology
University of Louisville School of Medicine

2008-present	Project 1: "AMP-activated protein kinase (AMPK) in the regulation of macrophage inflammatory function". This project has entailed the investigation of the mechanisms by which AMPK regulates inflammatory signaling and has resulted in the identification of AMPK as a critical component of the anti-inflammatory activity of IL-10 via its impact on PI3K/Akt/mTOR and STAT3/SOCS3 pathways.

2013-present Project 2: "Influence of myeloid-expressed AMPK on onset and progression of experimental autoimmune encephalomyelitis (EAE)".
I am currently a team member in the investigation of the role of myeloid-expressed AMPK in the regulation of EAE. Work in the lab has shown that AMPK activity in macrophages and dendritic cells strongly influences T cell differentiation during antigen presentation and thereby can influence both early events in development of autoimmune disease as well as the inflammation that occurs with disease progression.

2013-present Project 3: "AMPK regulation of macrophage function in tumor growth and metastasis"
I am currently a team member in the investigation of AMPK regulation of macrophage functional polarization in the tumor environment. This project is based on the hypothesis that AMPK contributes to the tumor-supportive function of tumor-associated macrophages.

Position: Research Assistant
Mentors: Gehong Wei, Ph.D., Professor (project 1); Zengqiang Zhang, Ph.D., Professor (project 2)
Northwest A & F University, Shaanxi, China

2007-2008 Project 1: "Sweet clover rhizobium genetic diversity and its role in nodule formation and nitrogen fixation".
We isolated rhizobium from the nodules of sweet clover plants sampled from northwest China. The bacteria's genetic diversity and the role in nodule formation and nitrogen fixation were analyzed.

2006-2007 Project 2: "Use of zero-valent iron for nitrate removal from the loess soil water in highland areas".
We sampled nitrate polluted loess soil water from highland areas of northwest China. The method of Permeable Reactive Barriers (PRB) based on zero-valent iron powder to effectively remove the nitrite pollution in the loess soil water was tested and optimized for practical use in highland areas of China.

2005-2006 Project 3: "Classification and cultivation of an unrecorded mushroom in Gansu Province".
We sampled a rare type of mushroom from cold, high latitude area of Gansu Province. Morphology studies were performed to identify the mushroom classification and the reserved spores were dedicated for indoor cultivation.

2005 Student trainee. "Instrumental operation skills training".
I was trained and certified to operate analytical instruments including Gas Chromatography (GC), High-Performance Liquid Chromatography (HPLC), Nuclear Magnetic Resonance (NMR), and Scanning Electron Microscope (SEM).

Position: Research Intern
Hanzhong Sci-Tech Bureau/Institute of Botany, Shaanxi, China

2005 I received training in general biotechnology lab skills as an undergraduate intern.

MEMBERSHIPS AND ACTIVITIES

2010-present *Member,* Society for Leukocyte Biology

2010-present *Member,* American Heart Association

2008-present *Member,* Microbiology and Immunology Student Organization, University of Louisville School of Medicine, Louisville, KY USA

2008-present *Member,* Graduate Student Council, University of Louisville School of Medicine, USA

2011 *Admissions Committee Representative,* Microbiology and Immunology Student Organization, University of Louisville School of Medicine, Louisville, KY USA

2003-2007 *Member,* Scientific Research Study Group, Northwest A & F University, China

HONORS AND AWARDS

10/29/2012 *Invited speaker and conference poster presenter*, Inflammation in Innate and Adaptive Immune Mechanisms, 45th Annual Meeting of The Society for Leukocyte Biology, Maui, Hawai'i, USA

2008-2010 *Awardee,* Integrated Programs in Biomedical Sciences (IPIBS) Fellowship, University of Louisville, Louisville, KY USA

09/2006 *Third place winner,* English Speech Contest, Northwest A & F University, China

2004 *Third place winner,* Social Practice Paper Competition, Northwest A & F University, China

2003-2007 *Third place winner,* Honored Student Scholarship, Northwest A & F University, China

PRESENTATIONS

10/21/2013 *Conference poster presenter*, Regulators of Innate Cell Plasticity, 46th Annual Meeting of The Society for Leukocyte Biology, Newport, Rhode Island, USA

10/29/2012 *Invited speaker and conference poster presenter,* Inflammation in Innate and Adaptive Immune Mechanisms, 45th Annual Meeting of The Society for Leukocyte Biology, Maui, Hawai'i, USA

09/23/2011 *Conference poster presenter,* Infection Inflammation Immunity, 44th Annual Meeting of The Society for Leukocyte Biology, Kansas City, USA

10/11/2011 *Poster presenter,* Research ! Louisville, University of Louisville, Louisville, KY USA

RESEARCH SUPPORT

2010-2012 American Heart Association, Great Rivers Affiliate Spring 2010 Predoctoral Fellowship (**PI Yanfang Zhu**)
"AMPK regulation of macrophage function in atherosclerosis"
Award Total $46,000

2005-2006 National Sci-tech Innovation Fund for College Students (one year fellowship), National Ministry of Education, China (**PI Yanfang Zhu,** Co-I P. Zhang, S. F. Zhang)
"Classification and cultivation of an unrecorded mushroom in Gansu province"

PUBLICATIONS

Peer Reviewed Manuscripts

Cilai Tang, **Yanfang Zhu**, Yingchao Zhang, Zengqiang Zhang. 2007. Use of zero-valent iron for nitrate removal from the soil water in loess areas [J]. *Acta Scientiae Circumstantiae* 27(8): 1292-1299. *This publication is in Chinese.*

Manuscripts Submitted

Yanfang Peipei Zhu, Jonathan R. Brown, Duygu Sag, Lihua Zhang, and Jill Suttles. 2014. AMP-activated protein kinase regulates IL-10-mediated antiinflammatory signaling pathways in macrophages.

Published Abstracts

Yanfang Zhu, Jonathan R. Brown, and Jill Suttles. 2013. AMPK is required for IL-10 activation of the PI3K/Akt/mTOR and STAT3/SOCS3 signaling pathways in macrophages. *J. Leuk. Biol.* S46 Abst.116

Yanfang Zhu, Jill Suttles. 2012. AMPK is required for IL-10 activation of the PI3K/Akt/mTOR and STAT3/SOCS3 signaling pathways in macrophages. *J. Leuk. Biol.* S22 Abst.52

Yanfang Zhu, Duygu Sag, and Jill Suttles. 2011. Elevated AMPK activity in Fatty Acid Binding Protein-deficient macrophages: evidence for AMPK maintenance of an IL-10 autocrine loop. *J. Leuk. Biol.* S40 Abst.116