The Role of Nutrient Sensing in Macrophage Polarization

Citation

Covarrubias, Anthony Joseph. 2016. The Role of Nutrient Sensing in Macrophage Polarization. Doctoral dissertation, Harvard University, Graduate School of Arts & Sciences.

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The role of nutrient sensing in macrophage polarization

A dissertation presented

by

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to

The Program in Biological Sciences in Public Health

in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in the subject of
Biological Sciences in Public Health

Harvard University
Cambridge, Massachusetts

December 2015

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The role of nutrient sensing in macrophage polarization

ABSTRACT

Macrophages are essential immune cells that belong to the innate immune system and are key orchestrators in the initiation and resolution stages of inflammation. Macrophages are found in every organ throughout the body where they function as sentinel cells that protect against invading pathogens and maintain tissue homeostasis by engulfing apoptotic cells. Thus, macrophages are able to efficiently recognize both pro- and anti-inflammatory stimuli and respond appropriately by activating a diverse set of biological programs. Over the last two decades, studies have shown that activated macrophages are able to polarize to pro-inflammatory or anti-inflammatory states with distinct phenotypes and physiological responses during inflammation. As a result, macrophages have been categorized into subtypes that include the classical pro-inflammatory M1 state, which is activated by Interferon-□and LPS and the "alternative" M2 state, which is activated by the Th2 cytokines IL-4 and IL-13.

Metabolic diseases, such as obesity and Type-II diabetes, are associated with chronic low-grade inflammation due to dysregulation of metabolic and inflammatory signaling pathways. During obesity monocytes infiltrate adipose tissue and polarize to M1 macrophages, displacing the M2 macrophages normally found in non-obese tissue. These M1 macrophages produce high levels of pro-inflammatory cytokines, which contributes to the development of insulin resistance and type-2 diabetes. Interestingly, while macrophages reside in all metabolic organs such as adipose tissue, how the nutrient status of the host affects macrophages function is not well understood. Thus, my thesis seeks to provide a clearer understanding of this important relationship and is of significant relevance to metabolic health and dysfunction.

Mammalian Target of Rapamycin (mTOR) lies downstream of TSC1/2 complex and plays a central role in integrating signals for energy sensing and other cellular processes such as cell proliferation, cell size, gene transcription, protein synthesis, and autophagy. mTOR exists in two separate complexes, mTORC1 and mTORC2, that regulate distinct signaling pathways and phosphorylation of downstream targets. Using a mouse model in which myeloid lineage specific deletion of Tsc1 (Tsc1$^{\Delta/\Delta}$) leads to constitutive mTOR Complex 1 (mTORC1) activation, we found that Tsc1$^{\Delta/\Delta}$ macrophages are refractory to IL-4 induced M2 polarization in vitro and in vivo, but produce increased inflammatory responses to proinflammatory stimuli such as LPS. The underlying basis for this aberrant macrophage polarization is due to feedback inhibition of Akt, a critical pathway that is activated by IL-4 and LPS. Thus, we elucidate a key role for the Akt pathway in macrophage polarization. In further studies of the Akt-mTORC1 pathway we found that IL-4 activation of the Akt-mTOR nutrient-sensing pathway leads to the phosphorylation and activation of the Akt target Acly (ATP-Citrate Lyase) that catalyzes the increase in cytosolic/nuclear pools of acetyl-CoA. This acetyl-CoA is used by histone acetyltransferases to acetylate the promoters of canonical M2 markers in murine macrophages such as Arg-1, Fizz1, and Mgl2. Furthermore, we have *in vitro* and *in vivo* evidence that macrophage M2 polarization can be tuned to metabolic input/nutrient status via the Akt-ACLY pathway and thereby control M2 gene expression levels. These findings highlight a key role for the Akt-mTORC1 pathway in regulating macrophage M2 polarization, and suggest how nutrient and metabolic status can fine-tune macrophage function via nutrient sensing pathways.

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Dedicated to Martin and Maria Almaraz and in loving memory of Antonio & Virginia Covarrubias

ACKNOWLEDGEMENTS

I would like to thank the Almaraz and Covarrubias family and longtime family friends (Daniel Godinez, Ricardo Ramirez, Nick Mendez, Frank Delgado and Felix Heredia) who have been a constant support. My best friend and partner Diane Ramirez for her companionship, support, and advice. Most of all I would like to thank my grandparents, my uncle Mitchell Almaraz, my aunt Allison Almaraz, my sisters, nieces and nephews, and my parents Tony and Melissa Covarrubias for their unconditional support and for always believing in me. I would also like to acknowledge my family members Carlos Lopez and Joe Castillo who passed away during my training but were always supportive and encouraged me to reach beyond my surroundings. My undergraduate friends and fellow Bruins (Fabian Rodriguez, Luis Perez, and Gerardo Morales) for their support, go Bruins! Lastly, thank you to all my friends and hard working people from the City of Gardena who kept me out of trouble and allowed me to see and accomplish more than I could ever imagine.

CHAPTER 1

Introduction

Adapted from:

Covarrubias AJ[1], Aksoylar HI[1], Horng T[1]. (2015) Control of macrophage metabolism and activation by mTOR and Akt signaling. Semin Immunol. 2015 Sep 7. Pii: S1044-5323 (15)00063-9.

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Abstract

Macrophages are pleiotropic cells that assume a variety of functions depending on their tissue of residence and tissue state. They maintain homeostasis as well as coordinate responses to stresses such as infection and metabolic challenge. The ability of macrophages to acquire diverse, context-dependent activities requires their activation (or polarization) to distinct functional states. While macrophage activation is well understood at the level of signal transduction and transcriptional regulation, the metabolic underpinnings are poorly understood. Importantly, emerging studies indicate that metabolic shifts play a pivotal role in control of macrophage activation and acquisition of context-dependent effector activities. The signals that drive macrophage activation impinge on metabolic pathways, allowing for coordinate control of macrophage activation and metabolism. Here we discuss how mTOR and Akt, major metabolic regulators and targets of such activation signals, control macrophage metabolism and activation. Dysregulated macrophage activities contribute to many diseases, including infectious, inflammatory, and metabolic diseases and cancer, thus a better understanding of metabolic control of macrophage activation could pave the way to the development of new therapeutic strategies.

Keywords

1. Introduction

Here we review the role of mTOR and Akt in control of macrophage activation and metabolism. We begin with an overview of mTOR and Akt signaling, followed by a discussion of their roles in macrophage activation as revealed by genetic models in which their activities are perturbed. The metabolic underpinnings of their control of macrophage activation are beginning to be unraveled and is a new and exciting area of research in the field, thus in the last sections, we discuss metabolic control of macrophage activation, and the potential role of the mTOR and Akt signaling in this process.

2. Overview of mTOR and Akt signaling

The serine threonine kinase mTOR is a key regulator of cellular metabolism that is conserved from yeast to man(1, 2). In mammals, mTOR exists in two complexes, mTORC1 and mTORC2 (Figure 1-1). Other subunits are unique to and define the two complexes, such as Raptor and Rictor in mTORC1 and mTORC2 respectively, and serve to regulate complex stability, activation, and/or activity. mTORC1 couples nutrient availability to major anabolic processes (Figure 1-1). In growing/proliferating cells, mTORC1 promotes the synthesis of macromolecules (e.g. lipids, proteins, and nucleotides), while in metabolic tissues like the liver, mTORC1 facilitates nutrient storage while inhibiting catabolic metabolism (e.g. autophagy). mTORC2 phosphorylates and activates Akt and other kinases of the AGC superfamily to control cellular metabolism, survival, and cytoskeletal organization (Figure 1-1). Interestingly, the activities of mTORC1, mTORC2, and Akt are intricately intertwined in some contexts. This includes growing and proliferating cells, in which Akt is a critical activator of mTORC1 and activated mTORC1 mediates feedback inhibition to shut down mTORC2 and Akt activation. Therefore, mTORC1, mTORC2, and Akt constitute a key metabolic signaling network that coordinates many metabolic processes that are best characterized in growing/proliferating cells and metabolic tissues (Figure 1-1)(1, 2).
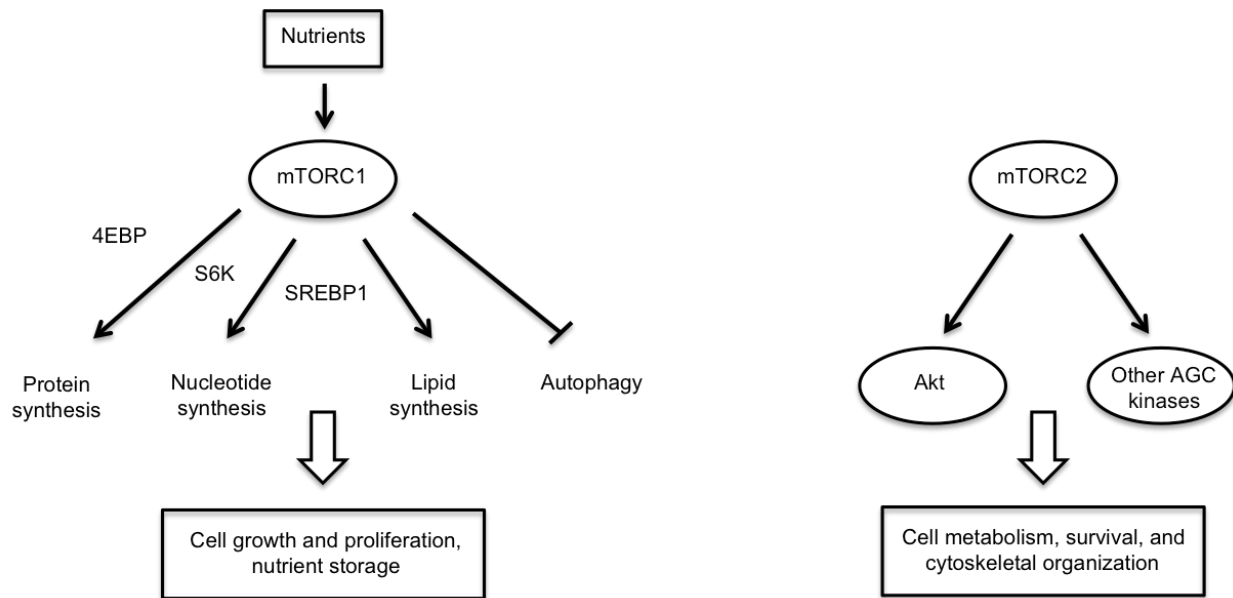
Figure 1-1. Overview of mTOR and Akt regulation and function. mTOR exists in two distinct complexes, mTORC1 and mTORC2. mTORC1 couples nutrient availability to anabolic processes such cell growth and proliferation and nutrient storage, by promoting the synthesis of macromolecules such as proteins, lipids, and nucleotides. mTORC2 phosphorylates Akt and other kinases of the AGC superfamily to regulate cell metabolism, survival, and cytoskeletal organization.

3. Activation of mTOR and Akt by macrophage polarizing signals

In contrast, the role of mTOR and Akt signaling in macrophages is much less well-understood. Importantly, emerging studies indicate that macrophage activities require the support of metabolic processes (see below). This suggests that polarizing signals that trigger macrophage activation should also induce metabolic shifts that support the acquisition and execution of relevant effector activities. Consistent with this idea, recent studies show that macrophage polarizing signals regulate mTOR and Akt signaling. We will discuss these studies below, which focus on M1 and M2 activation, but it is likely that most/all macrophage polarizing signals will impinge on major metabolic pathways to coordinate macrophage metabolism and activation.

Macrophage activation or polarization to the M1 (classical) and M2 (alternative) states has long served as a paradigm for studying macrophage activation(3, 4). During microbial infection, microbial stimuli such as LPS trigger M1 activation, which is characterized by increased production of pro-inflammatory cytokines and antimicrobial activity. M2 macrophages upregulate pro-fibrotic and tissue repair activities to coordinate Type 2 immunity, and are activated by stimuli such as IL-4 and IL-13 present during parasite infections (Figure 1-2). Induction of new effector activities by activated macrophages is regulated transcriptionally. Stat6 is the master regulator for M2 activation, while NF-kB and IRFs are key transcriptional regulators of the M1 program(3, 4).

In addition to these transcription factors, LPS and IL-4 signaling also target metabolic pathways such as mTOR and Akt to trigger metabolic shifts and metabolic reprogramming (Figure 1-2). In the case of IL-4, activation of Jak1 and Jak3 by ligation of the IL-4R allows for phosphorylation and activation of Stat6, as well as recruitment of the adaptor protein IRS2 (Figure 1-2A). IRS2 engages PI3K, which phosphorylates PIP2 at the plasma membrane to generate PIP3. PIP3 recruits Akt and mTORC2 to the plasma membrane, allowing mTORC2 to

phosphorylate and activate Akt. Activated Akt phosphorylates and inactivates the TSC complex. This complex, which contains TSC1 and TSC2, is a GAP for the small GTPase Rheb, so TSC inactivation results in Rheb activation. Although the underlying mechanism is not known, the GTP-bound form of Rheb activates mTORC1. Therefore, the IL-4R activates canonical signaling (e.g. Stat6) as well as the Akt-mTORC1 axis (Figure 1-2A)(5, 6). Of note, the Akt-mTORC1 axis is also engaged by growth factor signaling to regulate anabolic metabolism(1), and it is likely that this signaling module may have similar activities downstream of IL-4.

In the case of LPS-mediated M1 activation, TLR4 engages PI3K through an adaptor protein called BCAP (7), followed by Akt and mTORC1 activation (Figure 1-2B). The MEK/ERK(8) and IKKb (9) pathways have also been implicated in mTORC1 activation downstream of LPS, through their inactivation of the TSC complex. Moreover, a recent study in dendritic cells (DCs) suggests that Akt can be activated by a non-canonical mechanism, leading to Akt-mediated but mTORC1-independent regulation of aerobic glycolysis at acute time points after LPS stimulation. Such activation of Akt is independent of PI3K but dependent on TBK and IKKe (Figure 1-2B)(10). These findings indicate that regulation of Akt and mTORC1 downstream of TLR4 may be more complex compared to growth factor receptors and IL-4R, where an Akt-mTORC1 axis can be more clearly delineated.
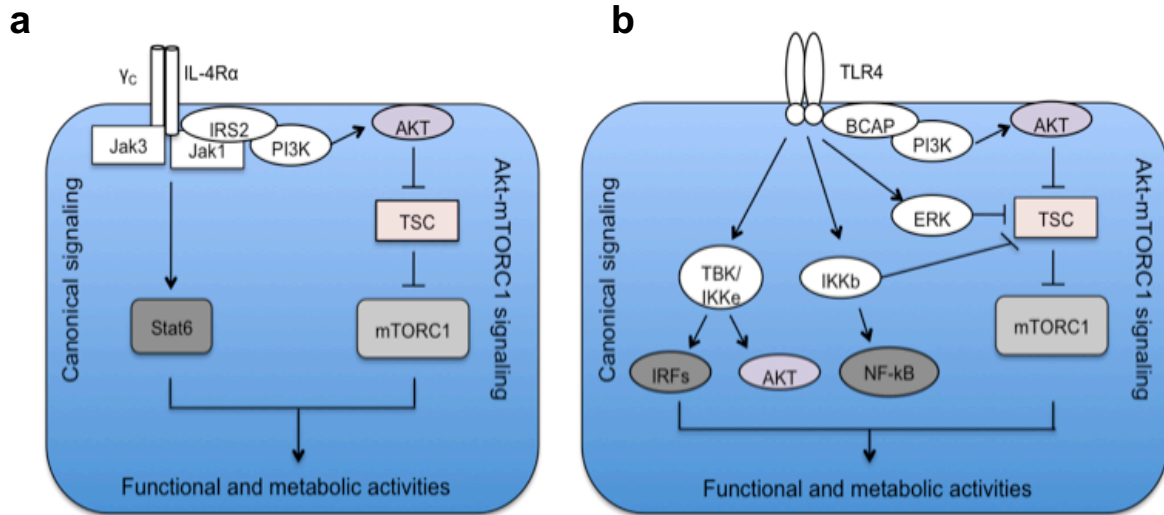
Figure 1-2. Integration of Akt and mTORC1 signaling into the IL-4R and TLR4 pathways.

A) In parallel to the canonical Jak-Stat pathway, the IL-4R activates the PI3K-Akt-TSC-mTORC1 signaling pathway.

B) TLR4 engages the adaptor protein BCAP to activate the PI3K-Akt-TSC-mTORC1 signaling pathway in parallel to the canonical pathway that culminates in activation of NF-kB and IRFs. Independent of its role in the PI3K-Akt-TORC1 pathway, Akt can be activated by canonical TLR4 signaling via TBK/IKKe. Additionally, other kinases activated by TLR4 signaling can regulate mTORC1 activity via phosphorylation of the TSC complex (e.g. Erk and IKKb). Such integration of Akt and mTORC1 signaling into canonical signaling allows for coordinate control of cellular metabolism and function during M1 and M2 activation.

4. Control of macrophage activation by mTOR and Akt signaling

In the next sections, we review studies indicating that Akt and mTOR play key roles in macrophage activation. Most studies to date have focused on how the mTORC1 and Akt pathways control either canonical signaling (e.g. JNK, NF-kB) or metabolic processes (e.g. HIF1α, glycolysis), thus we will discuss these topics separately, but future studies are expected to illuminate how control of the two processes are integrated.

Many studies have implicated a role for Akt in macrophage activation. Here we will briefly discuss some salient points, and the reader is referred to (11, 12) for additional reading. Akt appears to promote M2 polarization, since pharmacological inhibition of Akt attenuates induction of M2 genes(5, 13). Interestingly, only some M2 genes are regulated by Akt, and how specificity is achieved would be interesting to pursue in future studies. The role of Akt in M1 polarization is not quite as clear, with many studies implicating Akt and its upstream regulator PI3K as negative regulators, but others reporting positive regulation of M1 activation(12). Perhaps contributing to the discrepancy, the mammalian genome encodes 3 Akt proteins, of which Akt1 and Akt2 are thought to be expressed in macrophages. In one study, use of genetic models specifically deficient in one or the other Akt protein suggests that Akt1 inhibits while Akt2 promotes M1 activation, since loss of Akt1 and Akt2 augments and reduces M1 activation respectively (Table 1-1)(14). It is possible that depending on the experimental models used, preferential expression and/or activation of Akt1 versus Akt2 underlies the different results obtained with pharmacological Akt inhibitors (which are expected to target both proteins). Further analysis of macrophage activation using Akt1- and Akt2-deficient macrophages is warranted.

Akt is likely to control macrophage activation through multiple downstream effectors. For example, Foxo1, a transcription factor inhibited by Akt signaling, is implicated in proinflammatory gene induction in M1 macrophages(15). C/EBPβ is a transcription factor

Table 1-1; Macrophages with genetic ablation of mTORC1 and Akt reveal the critical roles of these metabolic regulators in control of macrophage activation. N.D., not determined.

	Macrophage polarization phenotype	Underlying mechanism	Metabolic phenotype	In vivo phenotypes
TSC-deficient BMDMs	Increased M1 [5,17,18]	Increased JNK activity [18], reduced Akt activity [5], increased Ras activity [17]	Defect in IL-4-inducible fatty acid oxidation [5]	Increased susceptibility to sepsis [18]; spontaneous development of inflammatory disorders [17]
	Reduced M2 [5,17]	Decreased Akt [5], decreased C/EBPβ [17]		Reduced IL-4 and chitin induced M2 activation [5]; resistant to asthma [17]
TSC-deficient DCs	Decreased M1 [20]; Mixed phenotype [19]	Reduced NF-kB activity [20]	Increased steady-state lipid synthesis and glycolytic metabolism [53]	Reduced T cell priming [19]
Rictor-deficient DCs and BMDMs (mTORC2 deficiency)	Increased M1 [26,27]	Relief of FoxO1 inhibition by Akt [27]		Increased susceptibility to sepsis [26]
Myeloid specific deficiency in Raptor (mTORC1 deficiency)	N.D.	N.D.		Resistance to obesity-associated complications [24] and atherosclerosis [25]
Akt1-deficient peritoneal macrophages	Increased M1 [14]	N.D.		Better survival in cecal ligation puncture (attributed to enhanced bacterial killing) but reduced survival in DSS colitis [14]
Akt2-deficient peritoneal macrophages	Reduced M1 [14]	Increased expression of C/EBPβ		Better survival in septic shock (attributed to decreased proinflammatory cytokines) and DSS colitis [14]

implicated in both M1 and M2 activation, and its expression has been shown to be controlled by Akt2 (Table 1-1)(14).Akt has also been implicated in positive as well as negative regulation of the activity of NF-kB(12), a master regulator of M1 activation.

Our discussion of control of macrophage activation by mTOR will be somewhat selective. Conflicting findings with respect to macrophage activation have been obtained with rapamycin, the small molecule inhibitor of mTORC1. In the mTOR field, it is well known that there are caveats associated with the use of rapamycin to inhibit mTORC1(16). Rapamycin is not a catalytic site inhibitor of mTOR and allosterically regulates mTOR activity in the mTORC1 complex through poorly understood mechanisms; such inhibition is selective in that some (e.g. S6K1) but not other (e.g. 4EBP1) targets of mTORC1 are affected. Additionally, while acute rapamycin treatment selectively inhibits mTORC1, prolonged treatment leads to mTORC2 disassembly and Akt inhibition. Catalytic site inhibitors of mTOR are available (e.g. Torin), but these will inhibit both mTORC1 and mTORC2. Therefore, analysis of mTORC1 activity solely by pharmacological blockade is problematic (16), and here we focus on studies where mTORC1 activity is perturbed by genetic models.

Several reports have addressed macrophage activation in macrophages genetically deficient in the TSC complex. Genetic models lacking Tsc1 or Tsc2, key subunits of the TSC complex, have been used to interrogate the consequences of aberrantly increased mTORC1 activity in multiple tissues and cell types, including liver, pancreas, and T cells. Mice with myeloid specific Tsc1 deficiency (LysM-Tsc1D/D) are born normally, but spontaneously develop inflammatory disorders, including inflammation in the liver and lung, and enlargement of the spleen and some lymph nodes(17). TSC-deficient bone marrow derived macrophages (BMDMs) appear to develop and differentiate normally in response to MCSF, but have elevated mTORC1 activity at basal state, as indicated by increased phosphorylation of its downstream targets S6K and 4EBP1. This contrasts with WT BMDMs, which have low basal mTORC1

activity that can be induced in a signal-dependent manner, for example by LPS or IL-4(5, 18).

Importantly, and consistent with the phenotype of LysM-Tsc1D/D mice, TSC-deficient BMDMs

mount a hyperinflammatory response to LPS(5, 18) but are deficient in IL-4-induced M2

activation (Table 1-1)(5, 17). Such divergence in M1 versus M2 activation is also observed with

other genetic models (e.g. deficiency of Stat6 or KLF4, transcription factors implicated in M2

activation), and reflects the opposing regulation of these two biological programs.

In terms of M2 activation, TSC-deficient BMDMs are attenuated in IL-4-mediated

induction of multiple M2 genes, including Arg1, Fizz1, and Ym1, as well as arginase activity(5,

17). In response to injection of IL-4 or chitin (which induces M2 activation in a IL-4-dependent

manner), peritoneal macrophages in LysM-Tsc1D/D mice are impaired in the induction of M2

genes. Likewise, these mice are resistant to OVA-induced allergic asthma—bronchoalveolar

lavage fluid macrophages from LysM-Tsc1D/D mice express lower levels of M2 markers,

correlating with reduced leukocyte infiltration into the lung and tissue pathology. Multiple

mechanisms may underlie diminished M2 activation in TSC-deficient BMDMs. One is

attenuated Akt signaling(5), a consequence of mTORC1-mediated feedback inhibition of

receptor proximal signaling events. While such negative feedback likely evolved to maintain

inducibility of the Akt-mTORC1 axis, increased mTORC1 activity in genetic models of TSC

deficiency severely blunts Akt activity. Consistent with a critical role for attenuated Akt signaling

in TSC-deficient BMDMs, ectopic expression of a constitutively active Akt mutant is able to

restore some features of M2 activation. Additionally, reduced expression of the transcription

factor C/EBPβ may underlie the polarization defect of TSC-deficient BMDMs. Akt activity has

been shown to regulate C/EBPβ expression(14), and ectopic expression of C/EBPβ rescues

the expression of some M2 genes in TSC-deficient BMDMs (Table 1-1)(17).

With regard to M1 activation, TSC-deficient BMDMs produce higher levels of pro-

inflammatory cytokines like TNF-a, IL-6, and IL-12 but lower levels of the anti-inflammatory

cytokine IL-10, at the mRNA and protein levels(5, 18). They have higher basal and LPS-inducible levels of the costimulatory molecules CD80 and CD86, and increased production of NO. The enhanced inflammatory phenotype is also observed with other TLR ligands, including PolyIC and Pam3Cys which stimulate TLR3 and TLR2 respectively, and with LPS and IFNg. In a LPS-induced endotoxin shock model, LysM-Tsc1D/D mice produce higher levels of inflammatory cytokines correlating with increased susceptibility (Table 1-1)(18). Although there is agreement on the exaggerated inflammatory response of TSC-deficient BMDMS, distinct and in some cases inconsistent underlying mechanisms have been proposed. Pan et al show that TSC-deficient BMDMs have increased JNK activity and that a JNK inhibitor reverses the enhanced NO production(18). Byles et al proposed that attenuated Akt activity contributes to increased inflammatory responses in TSC-deficient BMDMs(5). (As discussed above, constitutive mTORC1 activity leads to feedback inhibition of receptor proximal signaling events that reduces Akt activity.) Consistently, in some studies LPS-mediated induction of several pro-inflammatory cytokines is augmented by pharmacological Akt inhibition(11). Thus enhanced JNK and attenuated Akt activity may both contribute to the M1 phenotype of TSC-deficient BMDMs, and/or in a selective manner depending on the M1 effector response. Pan el at report that rapamycin treatment and/or deficiency of the mTORC1 subunit Raptor attenuates the hyperinflammatory phenotype of TSC-deficient BMDMs, indicating that the phenotype is a consequence of aberrant mTORC1 activity(18). In contrast, Zhu et al show that rapamycin treatment and deletion of mTOR do not reverse the phenotype of TSC-deficiency, and implicate Ras as a new TSC target(17). However, there are caveats associated with rapamycin treatment, and mTOR deletion would ablate mTORC2 as well as mTORC1 such that consequent reduction of Akt activity would lead to the observed increase in inflammatory gene expression; thus additional studies are warranted to definitively establish mTORC1 independency (Table 1-1). In contrast to a hyperinflammatory response in TSC-deficient

macrophages, TSC1-deficient DCs generated from the same mice have a more complex

phenotype characterized by increased production of some inflammatory cytokines but a

reduction in MHC class II expression and CD4 T cell priming(19). This may suggest cell-type

specific differences in the consequences of TSC-deficiency(19, 20) or alternatively, complexity

in regulation of the M1 program that should be addressed in future studies (Table 1). It is also

noteworthy that in CD8+ T cells, the degree of mTORC1 activity has been linked to quantitative

and qualitative differences in T cell responses(21, 22). Therefore, the onset, duration, and

strength of mTORC1 activity is likely to determine effects on macrophage metabolism and

activation.

Importantly, the enhanced M1 but diminished M2 activation of TSC-deficient

macrophages could be relevant to macrophage function in obesity. In the white adipose tissue,

polarization of adipose tissue macrophages (ATMs) to a M2-like state, mediated by IL-4 and IL-

13, is thought to be critical for maintaining tissue insulin sensitivity; during obesity, ATMs switch

to a M1-like phenotype, which directly promotes tissue inflammation, insulin resistance, and

metabolic derangement(23). Since the mTORC1 pathway is a major metabolic node that

integrates diverse metabolic inputs, metabolic signals may impinge on this pathway to modulate

ATM activation. Chronic nutrient excess may aberrantly activate mTORC1, leading to feedback

inhibition of Akt signaling and impaired responsiveness to IL-4 and IL-13; increased mTORC1

but decreased Akt activation in the ATM-containing stromal vascular fraction of mice on high fat

diet is consistent with this idea (A.C. and T.H., unpublished data). In contrast, how activation of

mTORC1 by physiological increases in metabolic signaling impacts M2 activation is less clear,

and it is possible that the consequence is to support certain aspects of ATM M2 activation. This

is also consistent with the role of the Akt-mTORC1 axis in insulin signaling—while postprandial

increases in insulin act in an Akt-mTORC1-dependent manner to stimulate nutrient storage in

the healthy liver, chronic nutrient excess leads to aberrant increases in mTORC1 signaling,

downregulated Akt activity, and hepatic insulin resistance(2). Likewise, physiological and pathophysiological Akt-mTORC1 signaling may have divergent outcomes in control of macrophage activation. Integration of the Akt-mTORC1 pathway into IL-4 signaling may allow this pathway to calibrate metabolic input to certain aspects of M2 activation, while corruption of the Akt-mTORC1 axis by chronic nutrient excess contributes directly to impaired macrophage polarization and loss of metabolic homeostasis. Additional studies are warranted to test this idea, which has implications for metabolic control of macrophage function in many contexts, most notably obesity-associated diseases like type 2 diabetes, atherosclerosis, and nonalcoholic steatohepatitis(23).

Studies analyzing macrophages deficient in Raptor or Rictor (and thus mTORC1 or mTORC2) are more limited. One study showed that mice with macrophage specific Raptor deficiency fared better on a high fat diet, with reduced liver and WAT inflammatory gene expression and increased systemic insulin sensitivity(24). Although not directly examined, this phenotype may be associated with increased M2 but decreased M1 gene expression in liver and WAT macrophages. Another study showed that Raptor deficiency in the myeloid compartment protected LDLR-/- mice from developing atherosclerosis on a western diet, as indicated by reduced lesion size and macrophage infiltration into plaques(25). These studies suggest that in the context of nutrient excess, Raptor signaling promotes pathophysiological M1 activation. Rictor deficient BMDMs and DCs have exaggerated M1 activation, expressing higher levels of inflammatory cytokines in response to LPS stimulation(26, 27). Consistently, myeloid specific deletion of Rictor leads to increased susceptibility to sepsis(26). Rictor deletion ablates mTORC2 assembly and Akt activation, thus the underlying defect may be due to reduced Akt activity. As mentioned above, Akt appears to antagonize many aspects of LPS signaling, and in Rictor deficient BMDMs, Akt-mediated inhibition of FoxO1 is alleviated, allowing the transcription factor to promote induction of inflammatory genes (27)(Table 1-1).

In summary, the studies above focused on regulation of "canonical" signaling (e.g. JNK and NF-kB) by mTOR and Akt in control of macrophage activation. Later in the review, we discuss emerging studies that highlight the metabolic aspects of mTOR- and Akt-regulated macrophage polarization.

5. Overview of macrophage activation and metabolism

As alluded to above, macrophages are pleiotropic cells that assume diverse functions depending on the context. M1 macrophages upregulate pro-inflammatory and antimicrobial activities, while M2 macrophages coordinate tissue repair and Type-2 immunity. Tissue-resident macrophages mediate tolerance to the gut microflora, insulin sensitivity in white adipose tissue, and thermogenesis in brown adipose tissue(3, 4). Emerging studies indicate that cellular metabolism and function are intricately intertwined, thus these diverse macrophage functions are likely to be supported by distinct metabolic programs. Here we present an overview of macrophage activation and metabolism. We discuss the types of signals that regulate macrophage metabolism and activation, and general principles underlying control of macrophage activation by metabolism.

In broad terms, there are two types of signals that regulate macrophage metabolism and function. First, metabolic signals, either systemic or derived from the tissue or microenvironment, can directly modulate macrophage activation and function. For example, fatty acids can play either a biosynthetic or regulatory role in stimulation of M2 activation. Fatty acids can engage β-oxidation, which supports M2 activation through unclear mechanisms, or activate nuclear receptors such as PPARγ and PPARδ, which synergize with Stat6 for transcriptional control of M2 activation(23, 28, 29). Another interesting example is regulation of two alternative macrophage activities in TB granulomas by availability of the essential nutrient oxygen. Macrophage iNOS uses oxygen as a substrate to produce NO and kill bacteria(30), but hypoxia in the necrotic core of the granuloma can upregulate macrophage expression of

Arginase. Arginase activity limits lung granuloma pathology, perhaps by local depletion of its

substrate Arginine and consequent suppression of T cell proliferation and activation(31).

Finally, lactate production by tumor cells undergoing Warburg metabolism skews tumor-

associated macrophages (TAMs) to an M2-like phenotype. Lactate stabilizes HIF1α expression

in the TAMs, allowing for induction of Arginase and pro-tumoral activities, perhaps via Arginase-

dependent production of polyamines that are essential for cellular proliferation(32).

Second, signals that are not strictly of metabolic nature can control macrophage

metabolism and activation. These signals include the prototypical polarizing signals such as

LPS and IL-4. As discussed above, macrophage activation requires metabolic support, hence

the need for polarizing signals to engage and regulate specific metabolic pathways. The

example of growth factor-mediated cellular proliferation is informative for illustrating why a

biological signal would need to trigger particular metabolic changes. Cellular proliferation

requires an accumulation of biomass in the form of proteins, lipids, and nucleotides. In

unicellular organisms like yeast, TORC1 couples nutrient availability to nutrient uptake and

macromolecule synthesis. In contrast, cellular proliferation in multicellular organisms is

regulated by growth factors, which allows proliferation to be coordinated with the needs of the

tissue and organism(1). Importantly, growth factors engage mTORC1 signaling such that

mTORC1 activity is controlled by both nutrients and growth factors; apparently growth factor

signaling co-opted the ancestral TORC1 pathway for regulating anabolic metabolism during the

evolution of multicellularity. Similarly, the signals that regulate macrophage activation engage

key metabolic pathways to trigger the metabolic shifts necessary to support the associated

functional reprogramming.

How might metabolic shifts control macrophage activation? First, metabolic shifts may

enable bioenergetic support of macrophage activities. Indeed, it has been suggested that the

distinct metabolism of M1 and M2 macrophages is necessary to meet their unique bioenergetic

requirements (33). M1 macrophages upregulate glycolytic metabolism, which allows for the rapid production of ATP that may be needed during infection by fast-replicating microbes. M2 macrophages augment β-oxidation, which is much more energy efficient (i.e. yielding more ATP) and thus more compatible with host defense to slow-growing and endemic parasites.

Second, metabolic shifts lead to corresponding changes in metabolites, both qualitative and quantitative. For example, increased glycolytic flux in M1 macrophages promotes the accumulation of lactate and succinate (resulting from aerobic glycolysis and pyruvate oxidation)(34, 35). In general, such changes to metabolite profiles can have a regulatory or biosynthetic role in controlling functional activities. Regulatory roles include modulation of signaling pathways, gene expression, and other cellular processes, since metabolites serve as essential cofactors or allosteric regulators of enzymatic activity. For example, succinate inhibition of prolyl hydroxylase (PHD) controls the stability of HIF1α, a transcription factor implicated in M1 activation(34) (see below). In a biosynthetic capacity, metabolites are substrates for protein modifications. This includes acetyl-CoA and S-adenosylmethionine, which are substrates for histone acetylation and methylation and thus control gene expression and epigenetics(36, 37). The biosynthetic roles of metabolites also support diverse macrophage effector activities. For example, ROS- and NO-mediated killing of intracellular bacteria is key to the antimicrobial activity of M1 macrophages. NADPH serves as an electron donor for both complexes, thus M1 macrophages boost production of this metabolite. As another example, LPS-stimulated DCs and macrophages produce high levels of inflammatory cytokines like TNFα, IL-6, and IL-12. This challenge to cellular secretory capacity is met by increased production of phospholipids, allowing for expansion of the ER and Golgi compartments(10).

In conclusion, macrophage activation is associated with metabolic shifts and metabolic reprogramming that enable bioenergetic and biosynthetic support as well as regulatory control of macrophage activities.

6. Metabolic control of macrophage activation

Here we discuss examples from recent studies indicating how metabolic shifts and metabolic reprogramming sustain macrophage activation. Because this topic has been comprehensively reviewed(38-40), we focus on the literature most relevant to mTORC1 and Akt. Diagrams of major metabolic pathways discussed in the text (Figure 1-3), as well metabolic control of M1 and M2 activation (Figure 1-4), are included for the reader's reference.

In M1 macrophages, LPS stimulation triggers a rapid increase in glycolytic flux via post-translational mechanisms (Figure 1-3). This is mediated, at least in part, by phosphorylation of hexokinase by activated Akt. Phosphorylated hexokinase associates with the mitochondria, which is thought to give the glycolytic enzyme privileged access to mitochondrial ATP to rapidly augment its activity and glycolytic flux(10). In other cell types, Akt upregulates endosomal recycling of Glut1 to augment cell surface expression of the glucose transporter(41), and it is likely that macrophage Akt mediates increased glucose utilization via multiple mechanisms. Transcriptional regulation underlies delayed, long-term boosts in glycolytic metabolism. Examples include induction of the glucose transporter Glut1 and glycolytic enzymes like Pfkfb3 and Ldha(35, 42). In some cases, alternative isoforms of metabolic enzymes are induced, apparently because their distinct activities play key roles in altering metabolic shifts (e.g. expression of the phosphofructokinase 2 isoform Pfkfb3 instead of Pfkfb1(42)). Transcriptional induction of many glycolytic enzymes is mediated by HIF1α, a master regulator of glycolysis that is activated by multiple mechanisms downstream of LPS signaling(35). One is via isoform switch to PKM2, the enzymatically less active isoform of Pyruvate kinase that doubles as a HIF1α -regulator, promoting its nuclear localization and transcriptional activity(43). A second

mechanism for HIF1α regulation is via increased oxidation of glucose-derived pyruvate, which

enhances TCA cycle activity leading to accumulation of the TCA cycle metabolite succinate.

Succinate inhibits the enzymatic activity of PHD, which mediates steady-state degradation of

HIF1α, so succinate accumulation stabilizes HIF1α(34, 43). In this way, the initial, Akt-mediated

increase in glycolytic flux is sustained by HIF1α-mediated transcriptional upregulation of the

glycolytic program (Figure 1-3 and 1-4A).

What are the consequences of increased glycolytic flux? As discussed above, one is

HIF1α stabilization, leading to transcriptional induction of glycolytic genes and a bona fide

reprogramming of the M1 macrophage to glycolytic metabolism. The gene encoding pro-IL-1β

is also regulated by HIF1α. Consistently, HIF1α-deficient macrophages are impaired in multiple

aspects of M1 activation, including induction of glycolytic genes and glucose uptake/metabolism

as well as IL-1β production(34, 35). Citrate is another TCA cycle metabolite that accumulates

upon LPS stimulation as a result of increased pyruvate oxidation in the mitochondria (Figure 1-

3). This enables citrate transport to the cytosol, where it serves as a precursor for fatty acid and

phospholipid synthesis, needed for expansion of the ER and Golgi compartments to

accommodate the increased secretory demand of M1 macrophages. Consistently, inhibition of

fatty acid synthesis reduces ER and Golgi expansion and secretion of inflammatory cytokines

like IL-6 and TNFα (10). Increased glycolysis in M1 macrophages also drives flux through the

PPP, which provides NADPH to fuel respiratory burst(44). Therefore, enhanced glycolysis in

M1 macrophages supports inflammatory cytokine production as well as respiratory burst. As in

the other examples above, transcriptional changes complement glycolytic flux in promoting

these metabolic processes, e.g., via transcriptional control of the genes encoding mitochondria-

to-cytosol transport of citrate (45) and flux through the PPP(44) (Figure 1-3 and 1-4A).
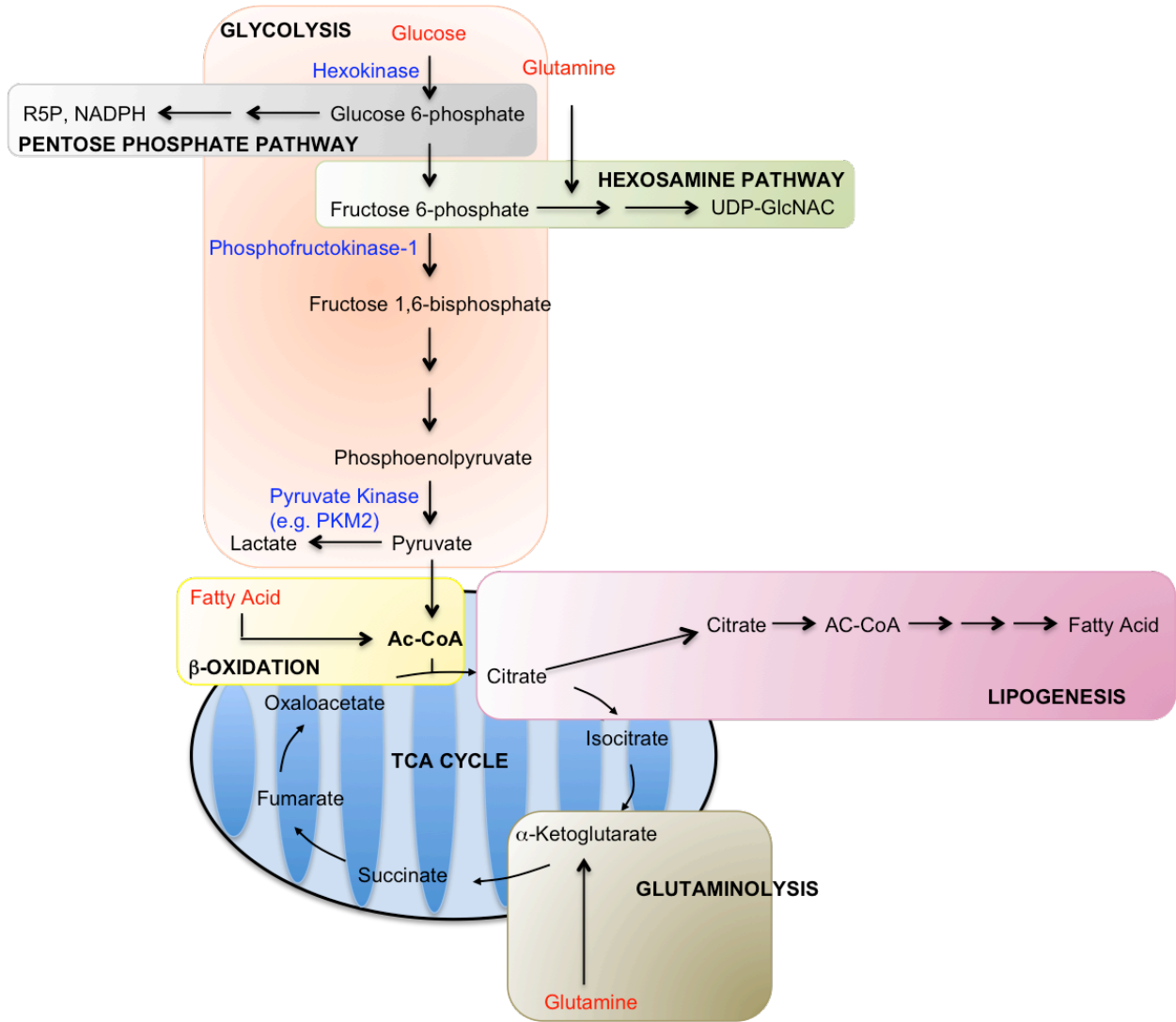
Figure 1-3. Overview of major metabolic pathways discussed in the text. Glucose is metabolized in the cytosol via glycolysis, yielding pyruvate which can be converted to lactate (Warburg metabolic or aerobic glycolysis). Alternatively, pyruvate can be further oxidized in the TCA cycle. The pentose phosphate pathway is a glycolytic shunt that produces ribose 5-phosphate and NADPH to support nucleotide synthesis and lipid synthesis. Glucose and

Figure 1-3 (continued)

glutamine also fuel the hexoamine pathway, which produces UDP-GlcNAC and other amino

sugars that are used for the generation of glycoproteins, glycolipids, and proteoglycans. Fatty

acids are catabolized via β-oxidation in the mitochondria, yielding Ac-CoA which enters the TCA

cycle. Glutamine is metabolized via glutaminolysis to produce a-ketoglutarate. Entry of α-

ketoglutarate into the TCA cycle ("anaplerosis") replenishes the cycle, which may be important

in conditions where citrate is diverted to the cytosol for Ac-CoA production and lipogenesis. The

TCA cycle is a major metabolic hub that produces cytosolic Ac-CoA (via citrate) for lipogenesis,

amino acids, and other key metabolites. In addition, NADH and $FADH_2$ generated by TCA cycle

activity fuels ATP generation by oxidative phosphorylation; alternatively, ATP can be produced

by aerobic glycolysis (not shown).
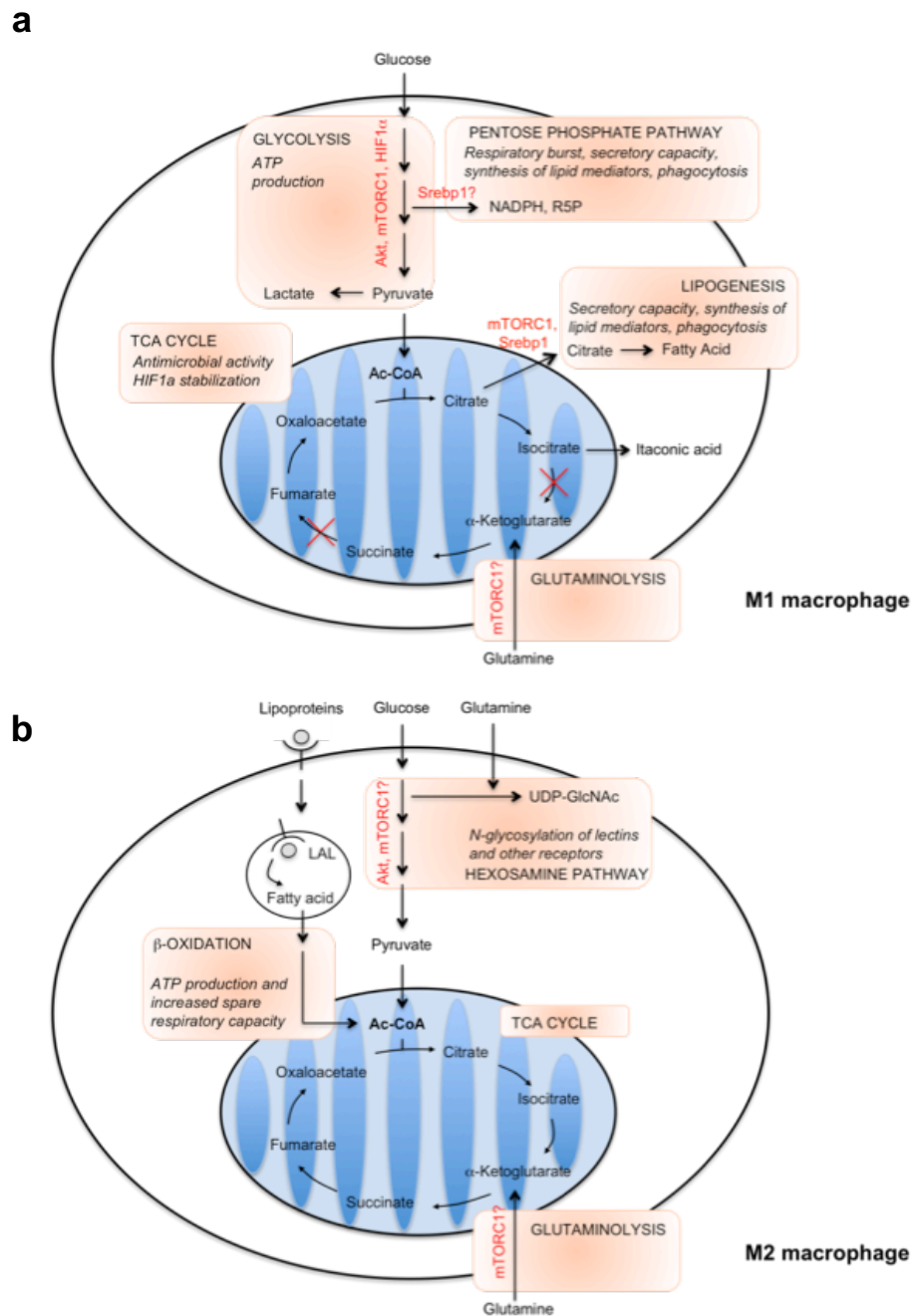
Figure 1-4. Control of metabolic nodes in M1 and M2 macrophages by mTORC1 and Akt.

A summary of the major metabolic shifts that occur in M1 and M2 macrophages.

Figure 1-4 (continued)

A-B). Metabolic shifts/processes are shown in boxes (uppercase font), as well as the

macrophage effector activity that is supported (italics). Nodes of regulation by mTORC1 and

Akt and their downstream effectors (HIF1α and Srebp1) are indicated (red). Question marks

indicate likely rather than established nodes of regulation.

As noted above, the LPS-stimulated increase in glycolytic flux initially promotes pyruvate oxidation in the mitochondria (enabling accumulation of TCA metabolites and enhanced oxidative phosphorylation), in addition to aerobic glycolysis (Figure 1-3). However, macrophages and DCs downregulate mitochondrial oxygen consumption after ~12 hours, due to damage of the electron transport chain by iNOS-mediated NO production(46). Related to this defect in oxidative phosphorylation, a recent study showed that M1 macrophages harbor "breaks" in the TCA cycle. One break is mediated by LPS-triggered downregulation of the TCA cycle enzyme isocitrate dehydrogenase(47). This leads to accumulation of the precursor isocitrate, which is used to produce itaconic acid, a metabolite with antimicrobial activity (Figure 1- 4A)(48). Therefore, a broken TCA cycle in M1 macrophages appears to be critically linked to antimicrobial activity.

In addition to an increase in glucose utilization, M1 macrophages upregulate glutamine metabolism(34, 47). Glutamine is metabolized to the TCA cycle metabolite α-ketoglutarate via glutaminolysis. Such replenishing of the TCA cycle ("anaplerosis") stimulates succinate accumulation and HIF1α activation (Figure 1-3 and 1-4A)(34). Pharmacological inhibition of glutamine metabolism reduces production of IL-1β in M1 macrophages, as well as serum IL-1β production and mice survival during Salmonella infection and sepsis(34, 47).

Relatively less is known about the IL-4-mediated metabolic shifts that occur in M2 macrophages. As indicated above, these macrophages are thought to be dependent on β-oxidation, since pharmacological block of this process with etomoxir attenuates transcriptional induction of the M2 program (33). Although little is known regarding how it supports M2 activation, β-oxidation is upregulated via IL-4-mediated transcriptional induction of the nuclear receptors PPAR-γ and PPAR-δ, and their coactivator PGC1β. In macrophages lacking these master regulators of fatty acid oxidation and mitochondrial biogenesis, IL-4-inducible β-oxidation and M2 gene expression are deficient (28, 29, 33). An important source of fatty acids for M2

macrophages is exogenous lipoproteins, which are taken up by the scavenger receptor CD36

and broken down in the lysosome by lysosomal acid lipase (LAL). In support, deletion or

pharmacological ablation of LAL or CD36 reduces mitochondrial oxygen consumption and M2

activation (Figure 1-4B)(49). Enhanced β-oxidation is linked to augmented spare respiratory

capacity (SRC)(49), indicating an increased ability to make ATP by oxidative phosphorylation.

Such increase in SRC may be important when macrophages are challenged with energy-

intensive tasks, or alternatively has been linked to longevity.

M2 macrophages also increase glutamine utilization(47). This enables biosynthesis of

UDP-GlcNAc, the substrate for N-glycosylation, in the hexosamine pathway (Figure 1-4B).

Consistently, block of N-glycosylation or the hexosamine pathway reduces cell surface

expression of N-glycosylated M2 markers. Glutamine utilization also fuels TCA cycle activity via

anaplerosis and thus presumably oxidative phosphorylation(47) (Figure 1-4B). Finally,

glutamine consumption supports optimal induction of multiple M2 genes, although the

underlying basis is not known. Finally, a recent study indicates that Myc is upregulated by IL-4

stimulation and controls M2 activation(50). Myc is key regulator of oxidative metabolism and

other metabolic processes in many contexts, warranting further scrutiny of its role in

macrophage activation.

7. Established and likely nodes of metabolic control by mTOR and Akt

Little is known regarding control of macrophage metabolism by mTORC1 and Akt. Here

we review their major effector activities in tumor cells and proliferating cells, before discussing

established as well as potential nodes of mTORC1- and Akt-mediated control of macrophage

metabolism (Figure 1-4 and Figure 1-5). As mentioned above, mTORC1 promotes the synthesis

of proteins, lipids, and nucleotides in growing and proliferating cells(1) (Figure 1-1). Its control

of protein synthesis is mediated by 4EBP and S6K, key regulators of translation, and lead to

ribosome biogenesis and a profound increase in protein synthetic capacity. mTORC1 triggers

lipogenesis by activating the transcription factors Srebp1 and Srebp2, which regulate expression of key enzymes in fatty acid and cholesterol synthesis respectively. In addition to rate-limiting enzymes in fatty acid synthesis, Srebp1 upregulates expression of enzymes in the pentose phosphate pathway (PPP), a glycolytic shunt. Increased flux through the PPP provides reducing power during fatty acid synthesis, justifying co-regulation of PPP and lipid synthesis enzymes by Srebp1 (Figure 1-5). mTORC1 stimulates nucleotide synthesis by increasing the activity of CAD (via S6K1), which catalyzes the first steps of pyrimidine synthesis. mTORC1-mediated activation of Srebp1 also contributes to nucleotide synthesis because ribose-5-phosphate, a building block in nucleotide synthesis, is produced in the PPP (Figure 1-1 and 1-3). In this way, mTORC1 coordinates the synthesis of the major macromolecules needed in growing and proliferating cells(1).

In M1 macrophages, de novo lipogenesis supports expansion of the ER and Golgi compartments, which has been linked to secretion of high levels of cytokines. Mechanistically, this requires citrate production as well as activation of Srebp1, the transcriptional regulator of lipogenesis (Figure 1-4A, 1-5). Consistent with its role in this setting, Srebp1 accumulates upon LPS signaling and its absence reduces fatty acid synthesis(51). As in tumor cells, Srebp1 is activated by mTORC1 in macrophages, as indicated by accumulation of activated Srebp1 in TSC-deficient BMDMs (T.H., unpublished data). Another macrophage effector activity likely to be critically fueled by increased lipid synthesis is phagocytosis. Phagocytic activity, which is augmented in activated macrophages to accommodate killing of microbial pathogens and dead cell clearance, requires an increase of cellular membranes. Lipid synthesis may also sustain production of pro-inflammatory and anti-inflammatory lipid mediators like prostaglandins and lipoxins respectively(45). Lipid mediators are synthesized and stored in lipid bodies, cytosolic

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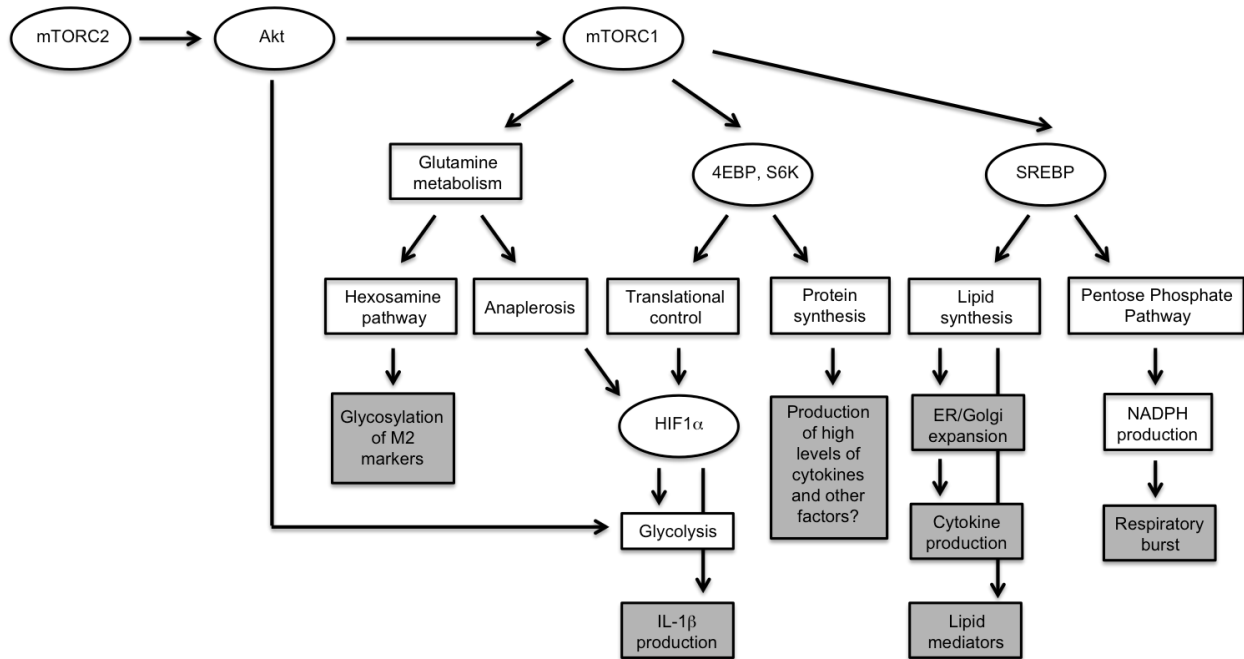
Figure 1-5. Schematic representation of control of macrophage activation by mTOR and Akt. Key metabolic regulators (ovals), metabolic pathways/processes (white rectangles), and macrophage effector activities (gray rectangles) are shown. In some cases, a regulatory link is inferred based on findings in other cell types (e.g. mTORC1-mediated translational control of HIF1α). See text for detailed discussion.

organelles that can be formed in response to macrophage polarizing signals in an mTORC1-dependent manner (52). It would be interesting to further explore the role of the mTORC1-Srebp axis in these macrophage effector activities. In addition, mTORC1-mediated increases in protein synthetic capacity is likely to be important for supporting the production of cytokines, chemokines, and other highly induced factors in activated macrophages. Whether nucleotide levels may be rate-limiting for transcription is not known, but mTORC1-mediated increases in nucleotide synthesis is thought to bolster protein synthesis in proliferating cells, because rRNA is an abundant component of ribosomes(1). Therefore, the ability of mTORC1 to promote protein and nucleotide synthesis may coordinate production of high levels of cytokines, chemokines, and other factors in activated macrophages, while lipid synthesis may support secretory capacity, phagocytosis, and production of lipid mediators (Figure 1-5).

mTORC1 and Akt regulate glycolysis in tumor cells and proliferating cells(1, 41), and apparently also in M1 macrophages. In response to LPS signaling, Akt mediates an initial increase in glycolytic flux (apparently independent of mTORC1), which is reinforced by HIF1α-mediated induction of the glycolytic program (Figure 1-4A, 1-5). In addition to the mechanisms described above for regulation of HIF1α activity (succinate accumulation and PKM2 induction), mTORC1 is likely to exert critical control. HIF1α levels are increased in TSC-deficient BMDMs (T.H., unpublished data), consistent with TSC-deficient MEFs where mTORC1 activity promotes translation of the HIF1α mRNA (Figure 1-5)(1). As discussed above, Srebp1 activation by mTORC1 is critical for lipid synthesis and boosts inflammatory cytokine production. Srebp1 also controls NADPH production in the PPP pathway(1), so the activity of the mTORC1-Srebp axis may underlie respiratory burst in M1 macrophages (Figure 1-4A, 1-5). Consistent with this discussion, TSC1-deficient DCs have increased lipid biosynthesis and glycolytic metabolism at steady state, although perturbed development and survival in this genetic model complicates analysis of responses to polarizing signals like LPS(53). Finally, mTORC1 has been linked to

glutamine metabolism through its control of glutamine hydrolysis to glutamate, the rate-limiting

step in glutamine utilization(54). As discussed above, M1 macrophages couple glutamine

consumption to anaplerotic refilling of the TCA cycle, which supports citrate production and

HIF1α stabilization (Figure 1-4A, 1-5).

mTORC1 and Akt could also support the metabolism of M2 macrophages. Here it is

worth reiterating that decreased M2 activation in TSC-deficient BMDMs is a consequence of

aberrantly increased mTORC1 activity, and that such mTORC1 hyperactivity is seen in

conditions of chronic nutrient excess and cancer(1). In our view, physiological mTORC1

signaling in macrophages may not antagonize M2 activation; rather, IL-4 signaling may engage

the Akt-mTORC1 pathway to support some aspects of metabolic reprogramming. For example,

Akt and mTORC1 regulate glucose utilization in other contexts and may stimulate UDP-GlcNAc

synthesis and N-glycosylation of lectins in M2 macrophages (Figure 1-4B). Another possible

node of control is glutamine metabolism, which is increased in M2 macrophages as discussed

above and is regulated by mTORC1 (Figure 1-4B). Additional studies are needed to test these

hypotheses.

A recent study has shed light on a new aspect of macrophage metabolism and activation

that is regulated by mTORC1 and Akt(55). Macrophages primed with a TLR ligand can be

rendered more responsive to a subsequent encounter, a process called "training". Macrophage

training may contribute to the ability of a primary challenge to protect against secondary

infection, for example during vaccination. Training is mediated by the Akt-mTORC1-HIF1α

pathway and a sustained upregulation of glycolysis that enable heightened responsiveness

upon the second challenge; blocking either glycolytic flux or the Akt-mTORC1-HIF1α pathway

during the initial challenge inhibits training. Importantly, training is reflected in epigenetic

changes at genes encoding glycolytic enzymes, and it would be important to determine how the

Akt-mTORC1-HIF1α axis coordinates such chromatin remodeling in future studies. Since

metabolites serve as cofactors of chromatin modifying enzymes and substrates of chromatin modifications, how regulation of metabolite levels by this signaling pathway would bear on chromatin regulation would be of interest. Such epigenetic control by mTORC1 and Akt signaling is likely critical to modulating macrophage functional states in vivo, given the long-lived nature of major macrophage populations in many tissues(55, 56).

8. Concluding remarks and future directions

As highlighted by the discussion above, mTORC1 and Akt (and metabolic processes more generally) can exert context-dependent effects in regulation of macrophage activation. M1 and M2 macrophages both increase glutamine utilization, but the metabolite is channeled into different metabolic pathways to support either HIF1α stabilization and IL-1β production or N-glycosylation of cell surface receptors. Likewise, mTORC1-dependent increases in lipid synthesis may support production of lipid mediators in all macrophages, but expression of Cox1 in M2 versus Cox2 in M1 macrophages allows for synthesis of anti-inflammatory and pro-inflammatory mediators respectively(40). A key determinant of these differences is differential expression of key metabolic enzymes, which is specified by the Jak-Stat pathway in M2 macrophages, or alternatively TLR signaling and downstream transcriptional regulators like NF-kB and IRFs in M1 macrophages. Therefore, mTORC1 and Akt work coordinately with the "canonical" pathway activated by the macrophage polarizing signal; canonical signaling provides specificity while the mTORC1 and Akt have a permissive but critical role, and together they ensure optimal macrophage activation and execution of effector activities (Figure 1-2).

Many questions remain outstanding in the emerging field of macrophage metabolism and activation and their control by mTORC1 and Akt signaling. A non-exhaustive list includes the following:

- How do polarizing factors other than LPS and IL-4 (e.g. IL-10, butyrate) regulate cellular metabolism to control macrophage activation?   In addition to mTOR and Akt, what other metabolic regulators are engaged by polarizing factors?

- mTORC1 is a major metabolic hub that integrates many stimuli. The TSC complex is regulated by cytokines, inflammatory stimuli, hypoxia, and energy levels, while amino acids control mTORC1 activity through an independent pathway. Do these diverse inputs converge on mTORC1 to regulate metabolic aspects of macrophage activation?

- Since macrophages assume a variety of functions depending on the tissue and context, it is logical to suggest that in vivo populations of macrophages are supported by distinct metabolic programs. It would be important to examine various macrophage populations in this regard, ideally complementing ex vivo metabolic profiling with in vivo analyses.

- Many pathogens exploit macrophages as a replicative niche, and host and pathogen modulate macrophage metabolism for antagonistic purposes during intracellular infection. While the macrophage seeks metabolic support for antimicrobial activities, the goal of the pathogen is to subvert host defense and to co-opt macrophage metabolism for its own survival and replication(57). Although the activities of mTORC1 and Akt underlie multiple aspects of host defense, macrophages inactivate mTORC1 during Shigella and Salmonella infection; mTORC1 inhibits autophagy, so this enables autophagic clearance of the bacteria. However, mTORC1 inactivation is transient during Salmonella infection, because the pathogen recruits mTORC1 to its vacuole to stimulate mTORC1 reactivation(58). Another example of bacterial modulation of macrophage metabolism is the M2-like metabolism enforced by *Salmonella typhimurium* and *Brucella abortus*, apparently to spare glucose for their own consumption(59, 60). Future studies will no doubt highlight pathogen exploitation of macrophage metabolism as a key component of host-pathogen interactions.

- Manipulation of metabolic pathways is being explored for therapeutic control of cancer and

  T-cell mediated diseases(61-64), and there is increasing interest in extending this to

  diseases where macrophages (and DCs) play a critical role. It is appealing to speculate that

  modulating macrophage metabolism can be used to enforce a switch in macrophage

  polarization that would be beneficial in sepsis, IBD, or cancer, for example. While

  specificity, efficiency, and safety are challenges associated with targeting macrophages by

  drugs or gene delivery, ongoing studies exploring the use of liposomes, nanoparticles, and

  other delivery systems may lead to viable therapeutic options(65, 66).

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CHAPTER 2

The mTOR pathway regulates macrophage polarization

Adapted from:

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David M. Sabatini[2], Brendan D. Manning[1], and Tiffany Horng[1]. (2013). The TSC-mTOR

pathway regulates macrophage polarization. Nature Communications. 2013; 4:2834.

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Abstract

Macrophages are able to polarize to proinflammatory M1 or alternative M2 states with distinct phenotypes and physiological functions. How metabolic status regulates macrophage polarization remains not well understood, and here we examine the role of mTOR (Mechanistic Target of Rapamycin), a central metabolic pathway that couples nutrient sensing to regulation of metabolic processes. Using a mouse model in which myeloid lineage specific deletion of Tsc1 ($Tsc1^{\Delta/\Delta}$) leads to constitutive mTOR Complex 1 (mTORC1) activation, we find that $Tsc1^{\Delta/\Delta}$ macrophages are refractory to IL-4 induced M2 polarization, but produce increased inflammatory responses to proinflammatory stimuli. Moreover, mTORC1-mediated downregulation of Akt signaling critically contributes to defective polarization. These findings highlight a key role for the mTOR pathway in regulating macrophage polarization, and suggest how nutrient sensing and metabolic status could be "hard-wired" to control of macrophage function, with broad implications for regulation of Type 2 immunity, inflammation, and allergy.

Introduction

Macrophages play a dynamic role in host defense and maintenance of tissue homeostasis. This necessitates a delicate balance between their proinflammatory and immunomodulatory functions to ensure appropriate responses to environmental stimuli. Macrophages can be broadly classified into M1 (classical) and M2 (alternative) subtypes based on function. M1 macrophages are activated by LPS and/or IFN-γ to elaborate proinflammatory cytokine production and tissue inflammation [1]. Conversely, M2 macrophages are stimulated by Th2 cytokines IL-4 and/or IL-13 to promote helminthic immunity, fibrosis, allergy, and immunomodulation [2]. Stimulation of macrophages with IL-4 and IL-13 leads to activation of the transcription factor STAT6, which is indispensable for M2 polarization [3]. Additionally, activation of the nuclear receptors PPARγ and PPARδ is necessary for full implementation of the M2 program [4, 5]. A hallmark of M2 macrophages is an increase in Arginase-1 gene expression and activity [2], which converts L-arginine to L-ornithine to promote polyamine synthesis and tissue repair [6]. The M2 program is also characterized by upregulation of C-type lectins, mannose receptor, chitinase family proteins, resistin-like molecules, and Interleukin-10, all of which contribute to immunomodulatory function [7]. Importantly, distinct metabolic programs are required to support energy demands of M1 and M2 macrophages. M1 macrophages rely primarily on glycolytic metabolism, mediated by HIF-1α, while M2 macrophages utilize fatty acid oxidation mediated by PPARγ and the transcriptional coactivator, PGC-1β [3, 8, 9]. This suggests that macrophage metabolism and inflammatory phenotype are integrally linked, and hint at additional regulatory control of macrophage polarization by metabolic pathways. The Mechanistic Target of Rapamycin (mTOR) is a key nutrient/energy sensor that couples nutrient availability to regulation of downstream metabolic processes such as protein synthesis, glycolysis, and de novo lipogenesis [10, 11]. mTOR, a serine/threonine kinase,

exists in a rapamycin-sensitive complex called mTORC1 that is negatively regulated by the tuberous sclerosis complex comprised of TSC1 and TSC2 [12] . Genetic loss of either TSC1 or TSC2 leads to constitutive mTORC1 activation [13] . Importantly, recent studies demonstrate that mTOR controls multiple aspects of T-cell biology including quiescence, activation, and differentiation [14] . However, little is known regarding the role of mTOR in regulating macrophage activation.

In the current study, we elucidate a role for mTOR in macrophage polarization. We demonstrate that $Tsc1^{\Delta/\Delta}$ macrophages have a marked defect in M2 polarization in response to IL-4, while the inflammatory response to LPS is enhanced. Aberrant polarization is due, at least in part, to mTORC1-mediated attenuation of Akt activity, which renders $Tsc1^{\Delta/\Delta}$ macrophages resistant to the immunomodulatory effects of Akt downstream of IL-4 and LPS signaling. Lastly, we show that IL-4 and chitin administration in $Tsc1^{\Delta/\Delta}$ mice recapitulates the defective M2 polarization in vivo.

Results

Constitutive mTORC1 Activity Impairs M2 Polarization

The mTOR pathway integrates a variety of inputs to regulate cell growth and to balance anabolic and catabolic processes [15] . Interestingly, stimulation of bone marrow derived macrophages (BMDMs) with IL-4 or LPS resulted in mTORC1 activation as indicated by increased phosphorylation of the downstream targets S6K1 and 4E-BP1 (Figure 2-1A), suggesting that the mTORC1 pathway may coordinate metabolic changes during macrophage activation. To examine this hypothesis, we utilized a model of myeloid- specific Tsc1-deficiency in which mTORC1 is constitutively active. $Tsc1^{loxP/loxP}$ LysMCre mice and $Tsc1^{loxP/loxP}$ controls are herein referred to as$Tsc1^{\Delta/\Delta}$ and $Tsc1^{fl/fl}$ respectively. Immunoblotting confirmed that TSC1 is absent in the $Tsc1^{\Delta/\Delta}$ BMDMs (Figure 2-1B). TSC2 protein level was also
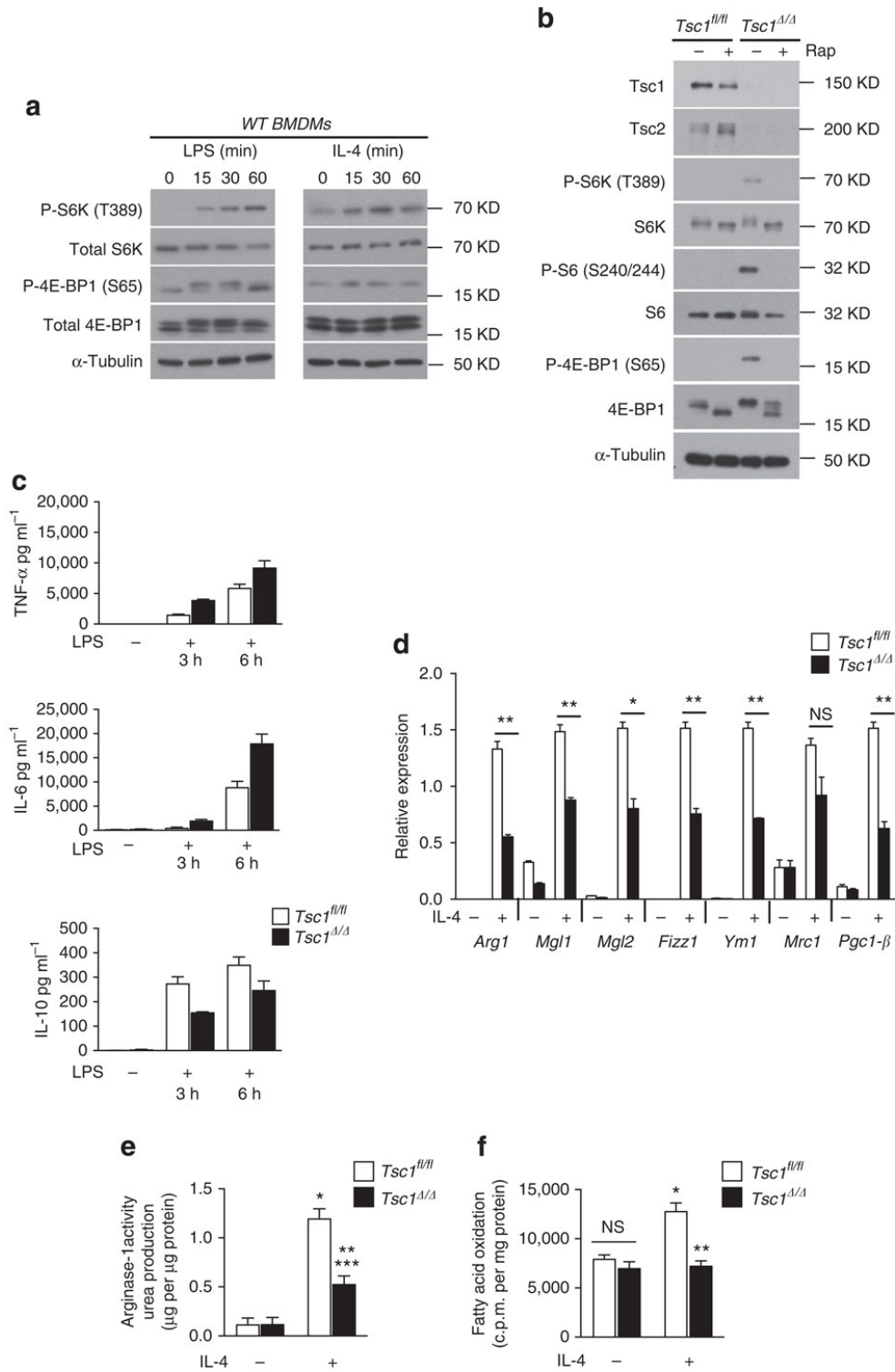
Figure 2-1. Tsc1$^{\Delta/\Delta}$ BMDMs Have Defective M2 Polarization and Enhanced Responses to LPS stimulation

Figure 2-1 (continued)

A) Immunoblot analysis of WT BMDMs stimulated with LPS or IL-4 for 15-60 min as indicated. (VB)

B) Immunoblot analysis of lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs treated with or without rapamycin for 15h. (VB)

C) Measurement of TNF-α, IL-6, and IL-10 secretion by ELISA after treatment with LPS for 3h and 6h, (n=2 representative experiments). (AC)

D) Expression of M2 genes in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs after treatment with IL-4 for 24h (n=3). *$p<0.05$, **$p<0.01$, ***$p<0.001$. (AC)

E) Urea production normalized to total protein in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated as in (c), (n=4), *$p<0.001$ for untreated vs IL-4 for Tsc1$^{fl/fl}$, **$p<0.01$ for IL-4 treated Tsc1$^{fl/fl}$ vs Tsc1$^{\Delta/\Delta}$, ***$p<0.05$ for untreated vs IL-4 for Tsc1$^{\Delta/\Delta}$. (AC)

F) Fatty acid oxidation of ^{3}H-palmitic acid presented as counts per minute normalized to mg of total protein after 36h treatment with IL-4, (n=3). *$p<0.01$ for untreated vs IL-4 in Tsc1$^{fl/fl}$, **$p<0.01$ for IL-4 treated Tsc1$^{fl/fl}$ vs Tsc1$^{\Delta/\Delta}$. Graphs are shown as mean ± SEM. P-values were determined using Student's t-tests. (VB)

diminished (Figure 2-1B), as TSC1 stabilizes TSC2 [16] . Tsc1$^{\Delta/\Delta}$ BMDMs displayed

constitutive phosphorylation of the mTORC1 downstream targets S6K1 and 4E-BP1, as well as

the S6K1 target ribosomal S6, all of which were sensitive to the mTORC1-specific inhibitor,

rapamycin (Figure 2-1B). Furthermore, Tsc1$^{\Delta/\Delta}$ BMDMs appeared to differentiate normally in

vitro and expressed similar levels of the macrophage markers F4/80 and CD11b (Figure 2-2).

As reported in other models of TSC- deficiency [17]. Tsc1$^{\Delta/\Delta}$ BMDMs were larger (Figure 2-2)

due likely to a role of mTORC1 in regulating cell size [18] . These observations confirmed

constitutive mTORC1 activation in Tsc1$^{\Delta/\Delta}$ BMDMs and established the validity of our genetic

model.

To assess macrophage polarization in Tsc1$^{\Delta/\Delta}$ BMDMs, we used LPS treatment to

promote an M1-like phenotype and IL-4 stimulation to induce an M2 phenotype. We found

that LPS-treated Tsc1$^{\Delta/\Delta}$ BMDMs secreted more of the proinflammatory cytokines IL-6 and

TNF-α, but less of the anti-inflammatory cytokine IL-10 (Figure 2-1C). Given the enhanced

responses of Tsc1$^{\Delta/\Delta}$ BMDMs to LPS, we postulated that M2 polarization could be

defective. Indeed, Tsc1$^{\Delta/\Delta}$ BMDMs failed to fully upregulate the M2 program, with significant

reductions in Arg-1, Mgl1, Mgl2, Ym1, Fizz1 and Pgc1-β expression (Figure 2-1D). Arginase-

1 activity assessed by urea production was reduced, correlating with lower levels of Arg-1

mRNA (Figure 2-1E). IL-4-stimulated fatty acid oxidation, another hallmark feature of M2

macrophages, was also defective (Figure 2-1F). This finding is consistent with the 3-fold

reduction in Pgc1-β (Figure 2-1D), a known mediator of fatty acid oxidation in M2

macrophages [9]. Interestingly, M2c polarization triggered by IL-10 stimulation was also

deficient in Tsc1$^{\Delta/\Delta}$ BMDMs (Figure 2-3A), which may indicate impaired orchestration of
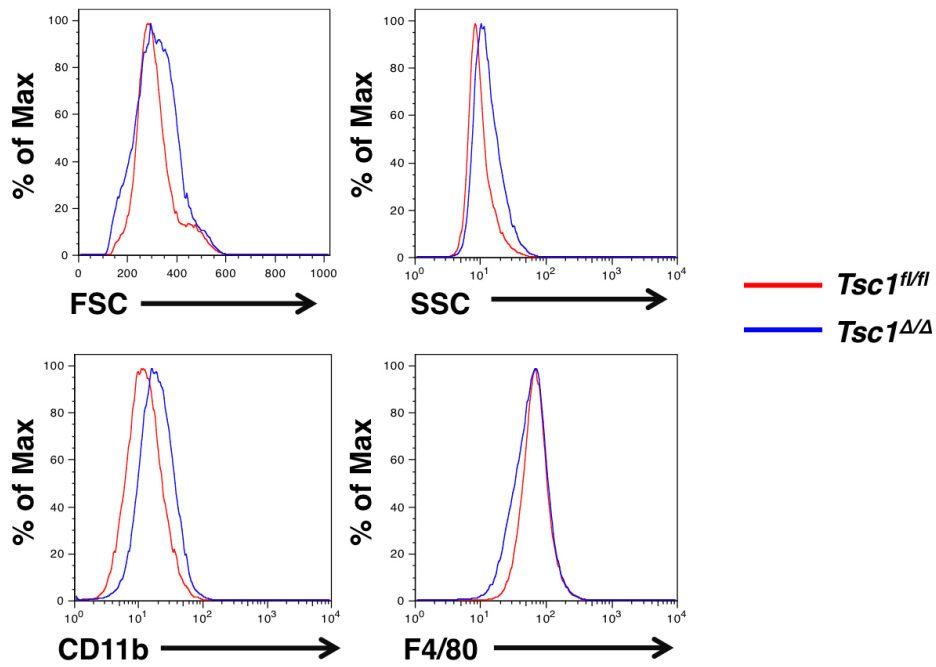
Figure 2-2. Increased Cell Size and Granularity in Tsc1$^{\Delta/\Delta}$ BMDMs. Flow cytometry

analysis of Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stained with macrophage markers CD11b and F4/80.

Histogram plots are shown as % of maximum. Red=Tsc1$^{fl/fl}$and Blue= Tsc1$^{\Delta/\Delta}$. (VB)

anti-inflammatory responses during tissue remodeling or wound healing [19]. Macrophage activation to the M2b phenotype (by treatment with LPS/immune complexes), which has features of both M1 and M2 macrophages [20, 21] , was not affected (Figure 2-3B). Taken together, our findings demonstrate that aberrant mTORC1 activation critically modulates macrophage polarization. Impaired induction of Pgc-1β and fatty acid oxidation also highlights a key role of mTOR in orchestrating macrophage cellular metabolism.

STAT6 and PPARγ Activity are Normal in Tsc1$^{\Delta/\Delta}$ BMDMs

IL-4R signaling leads to activation of JAK1/JAK3 and tyrosine phosphorylation of the transcription factor STAT6, enabling nuclear translocation [2] and induction of target genes such as Pparγ and Arg-1 [3] . To interrogate the mechanism underpinning defective M2 polarization in Tsc1$^{\Delta/\Delta}$ BMDMs, we first examined signal transduction downstream of the IL-4R. Tsc1$^{\Delta/\Delta}$ BMDMs expressed comparable levels of Jak1, Jak3, and IL-4rα mRNAs as well as STAT6 protein at basal state (Figure 2-4A and Figure 2-5A). Following IL-4 stimulation, STAT6 was tyrosine phosphorylated consistent with normal activation (Figure 2-4A). Chromatin immunoprecipitation assays indicated comparable IL-4- induced recruitment of STAT6 to the promoter of the Arg1 gene (Figure 2-5B). Furthermore, IL-4-inducible STAT6 transcriptional activity as measured by a STAT6 reporter assay was unaffected (Figure 2-4B). Finally, normal induction of some M2 genes inTsc1$^{\Delta/\Delta}$ BMDMs, including the STAT6-dependent gene Pparγ [22] (Figure 2-4C), indicates no general defect in expression or activation of STAT6, the master regulator of M2 gene induction. IL-4 signaling can activate STAT1 in some contexts [23, 24], but not in macrophages (Figure 2-5C) consistent with a prior report, excluding a role for impaired STAT1 activation in the phenotype of Tsc1$^{\Delta/\Delta}$ BMDMs [25]. Because previous studies indicate a key role for the nuclear receptors PPARγ and PPARδ in M2 polarization, we next turned to an analysis of their expression and activity in Tsc1$^{\Delta/\Delta}$ BMDMs. First, we
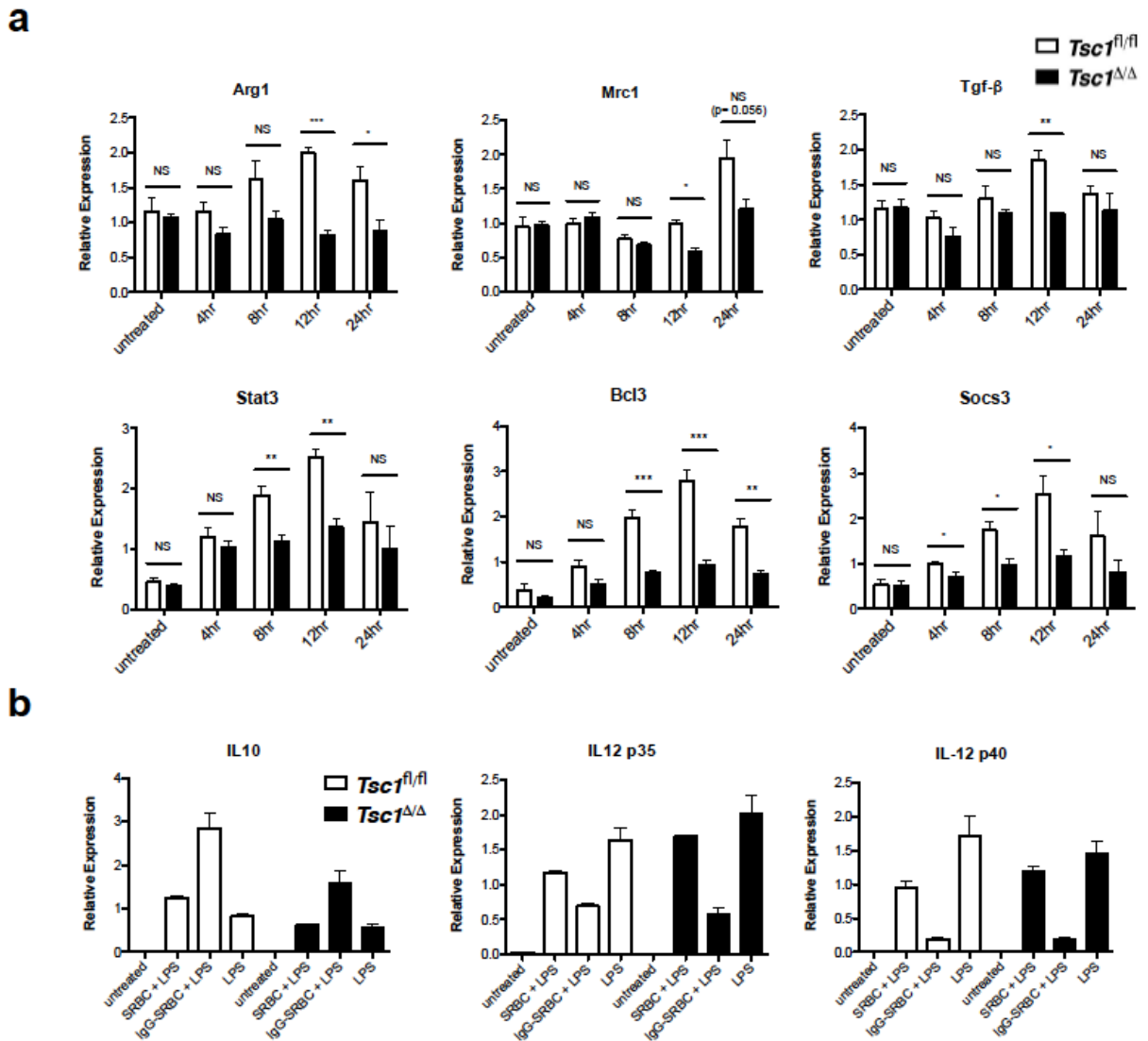
Figure 2-3. Impaired M2c but not M2b Polarization in Tsc1$^{\Delta/\Delta}$ BMDMs

A) M2c gene expression in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with 10ng/ml IL-10 for the indicated times (n=2 representative experiments). (AC)

B) M2b gene expression of Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated simultaneously with LPS and non-opsonized (SRBC) or opsonized SRBC (IgG- SRBC) for 6h (Data representative of 3 independent experiments). Graphs are shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 determined by Student's t-tests. (AC)

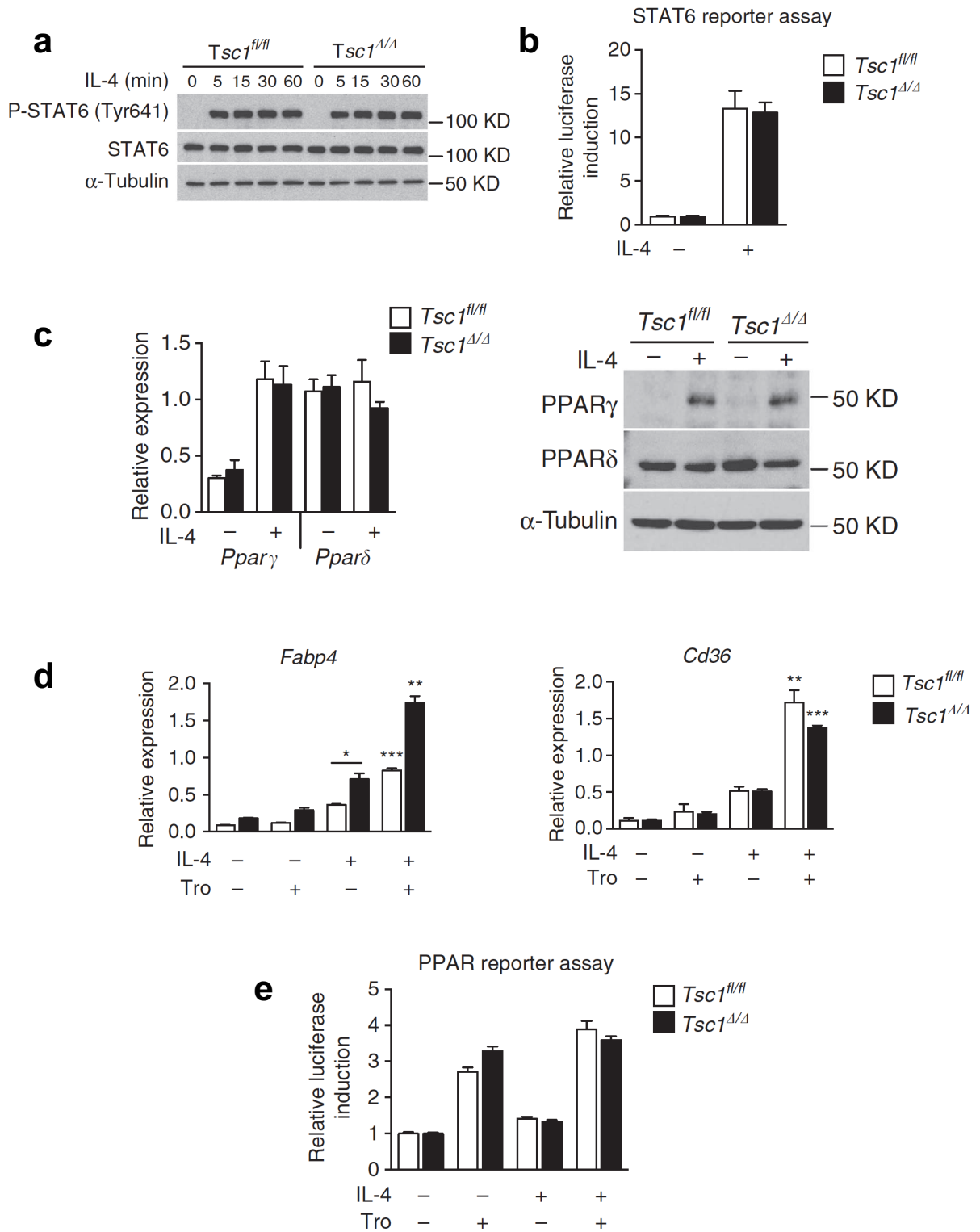**Figure 2-4. STAT6 and PPARγ Activity are Normal in Tsc1$^{\Delta/\Delta}$ BMDMs**

Figure 2-4 (continued)

A) Immunoblot analysis of lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 for 5–60min. (VB)

B) STAT6 luciferase reporter assay in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs. Data shown as fold induction of firefly luciferase activity normalized to renilla luciferase for IL- 4 treatment relative to untreated sample (n=2 experiments performed in duplicate). (AC)

C) Gene expression and immunoblots for PPARγ and PPARδ in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs after treatment with IL-4 for 24h. Gene expression data is shown as mean ± SEM (n=3). (VB)

D) Expression of PPARγ-dependent genes in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs treated with IL-4 in the presence or absence of troglitazone for 24h. DMSO vehicle was used as control, (n=3). *p<0.05 for IL-4 treated *Tsc1fl/fl* and *Tsc1Δ/Δ*, **p<0.01, ***p<0.001 for IL-4 versus IL-4+Tro. (VB)
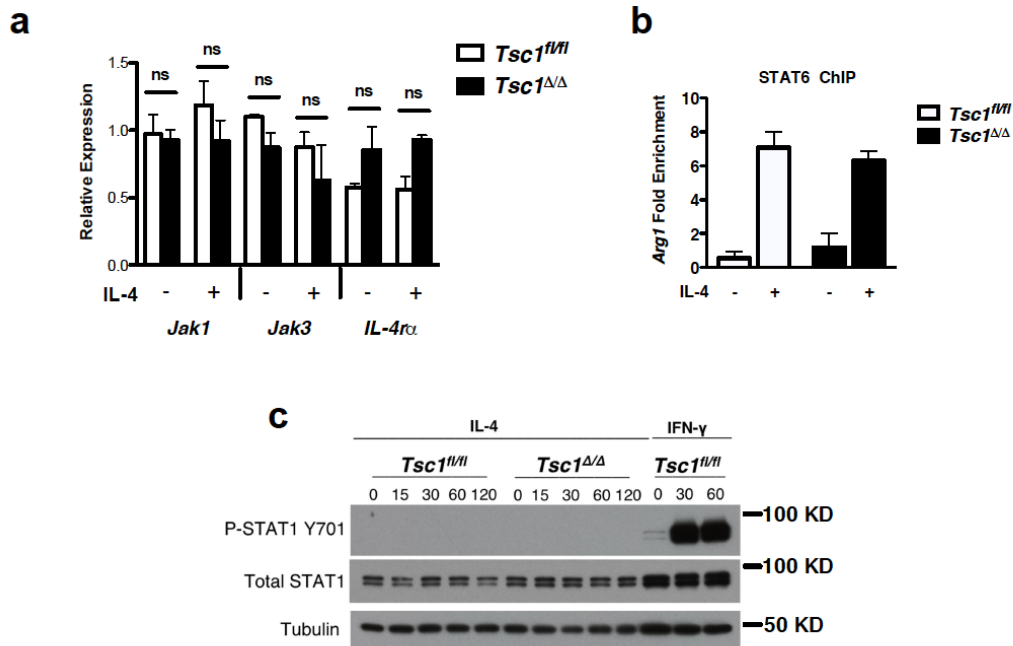
E) PPAR luciferase reporter assay in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs. Data shown as fold induction of firefly luciferase activity normalized to renilla luciferase for IL-4 or troglitazone treatment relative to untreated sample (representative of 3 experiments performed in triplicate). Graphs are shown as mean ± SEM. P-values determined using Student's t-tests. (AC)

Figure 2-5. The JAK-STAT6 Pathway is Intact in Tsc1$^{\Delta/\Delta}$ BMDMs

A) Gene expression of components of the IL-4R-JAK-STAT6 pathway (n=2 representative experiments). (AC)

**B)** STAT6 chromatin immunoprecipitation. Data is shown as fold enrichment for the Arg1 promoter and is representative of 3 independent experiments. (VB)

C) IL-4 fails to activate STAT1 in BMDMs. Immunoblot analysis of STAT1 activation in lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 or IFN (control) for the indicated time points. Graphs are shown as mean ± SEM. (AC)

showed comparable expression of PPARγ and PPARδ in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs, at

basal state and after IL-4 stimulation (Figure 2-4C). Since expression does not necessarily

reflect functional activity, we examined PPARγ activity in Tsc1$^{\Delta/\Delta}$ BMDMs. We found that IL-4

induced comparable expression of the canonical PPARγ-dependent genes Fabp4 [22, 26] and

Cd36 [27] (Figure 2-4D). Furthermore, the PPARγ agonist troglitazone synergized with IL-4 to

a similar extent in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs (Figure 2-4D). Analogous findings were

obtained with the PPARδ-dependent gene Atgl [28] using the PPARδ agonist GW501516

(Figure 2-6). To corroborate gene expression data, we used a PPAR reporter assay to assess

PPAR transcriptional activity, and found commensurate induction in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$

BMDMs (Figure 2-4E). Taken together, these findings indicate a selective defect in M2

polarization in Tsc1$^{\Delta/\Delta}$ BMDMs that may not be due to defects in STAT6, PPARγ, or PPARδ

expression or activity.

mTORC1 Activity Attenuates IL-4-Induced Akt Activation

The data above suggests that Tsc1$^{\Delta/\Delta}$ BMDMs may not be able to activate some

signals downstream of IL-4R. We turned our attention to IRS2/PI3K/Akt signaling, since this

pathway is engaged by the IL-4R in parallel to the JAK/STAT6 pathway [29, 30]. PI3K

activation leads to increased activity of mTORC2, which phosphorylates Akt at S473 to activate

the protein and promote membrane localization. In addition to S473, Akt is critically controlled

by phosphorylation at T308, a step mediated by PDK1 (Figure 2-7A). Thus we examined S473

and T308 phosphorylation as readouts of Akt activity, as well as phosphorylation of the

downstream Akt targets FOXO1, PRAS40 and GSK-3 [31] (Figure 2-7A). Interestingly, IL-4-

stimulated Tsc1$^{\Delta/\Delta}$ BMDMs displayed a striking attenuation in Akt signaling as indicated by

reduced phosphorylation of Akt^{S473} and Akt^{T308} (Figure 2-7B). Consistently, phosphorylation
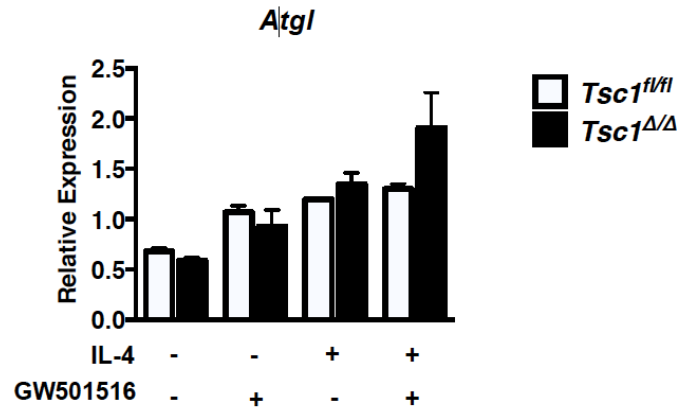
Figure 2-6. PPARδ Activity is Normal in Tsc1$^{Δ/Δ}$ BMDMs. Gene expression of Atgl in

Tsc1$^{fl/fl}$ and Tsc1$^{Δ/Δ}$ BMDMs stimulated with IL-4 in the presence or absence of GW501516.

DMSO vehicle was used as control. Data is shown as mean ± SEM (n=2 representative

experiments). (VB)
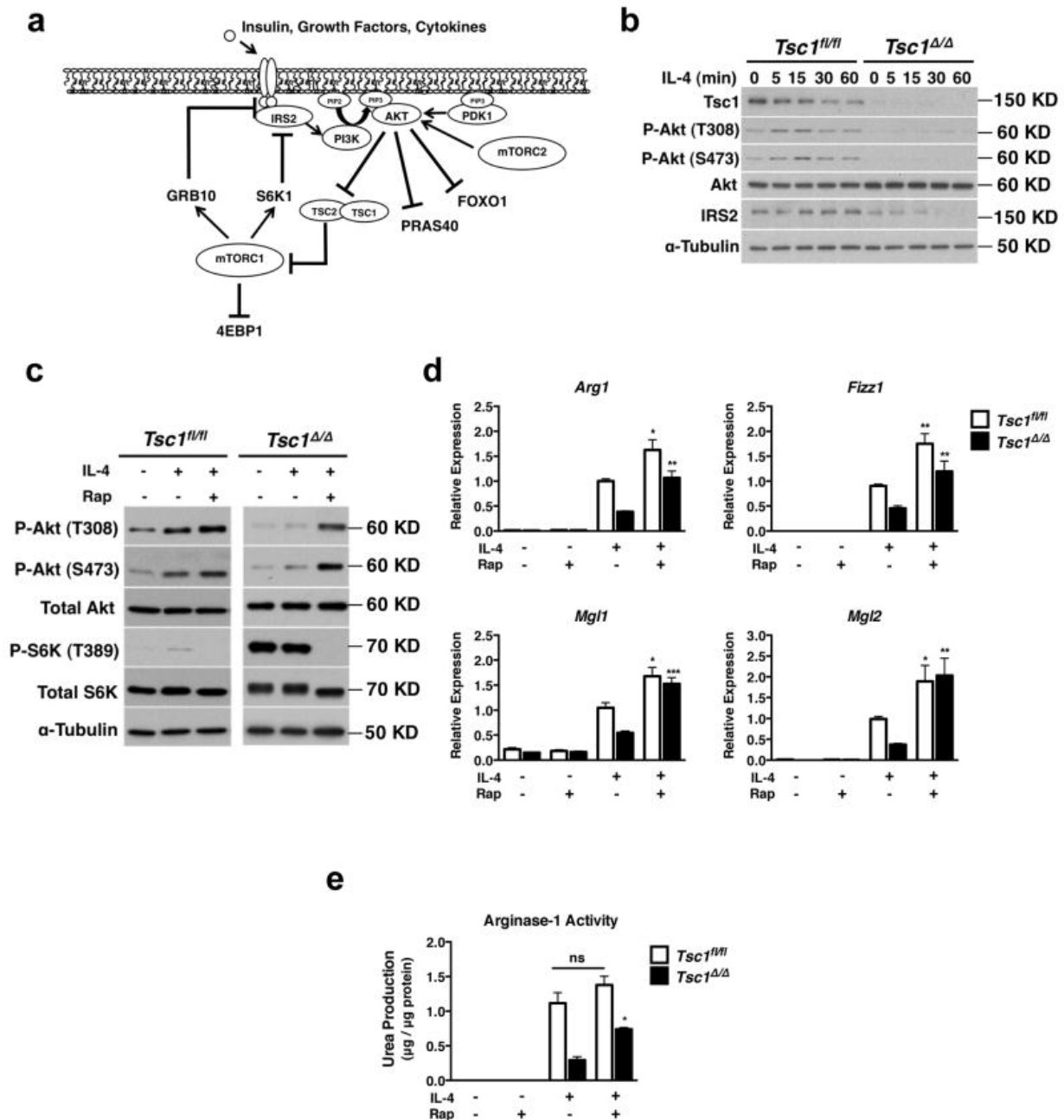
Figure 2-7. Constitutive mTORC1 Activity Attenuates IL-4-Induced Akt Activation

A) Overview of mTORC1 signaling downstream of IL-4, insulin, and growth factors. Receptor

activation engages the IRS/PI3K/Akt pathway. PI3K converts PIP2 to PIP3 thus recruiting

PDK1 and Akt to the plasma membrane, enabling PDK1-mediated phosphorylation of Akt on

Figure 2-7 (continued)

T308. PI3K also activates mTORC2, which phosphorylates Akt on S473. Thus activated, Akt can phosphorylate downstream targets to regulate their activity. One consequence of Akt activation is increased mTORC1 activity, which feeds back to attenuate IRS2/PI3K/Akt signaling through multiple mechanisms, including reducing levels of IRS2 while increasing levels of GRB10.

B) Immunoblot analysis of lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 for 5–60 min. (VB)

C) Immunoblot analysis of lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 for 20 min in the presence or absence of rapamycin (20nM, 1h pretreatment). DMSO vehicle was used as control. (VB)

D) Expression of M2 genes in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs after treatment with IL-4 for 15h in the presence or absence of rapamycin (20nM, 1h pretreatment). (n=5). $p<0.05$, $p<0.01$, $p<0.001$ for IL-4 versus IL-4+rap. (VB)

E) Urea production normalized to total protein in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL- 4 for 20h in the presence or absence of rapamycin (20nM, 1h pretreatment). (n=5),$p<0.001$. Graphs are shown as mean ± SEM. P-values were determined using Student's t-tests. (VB)

of the Akt targets FOXO1 and PRAS40 was diminished (Figure 2-8A). GSK-3

phosphorylation was not reduced (Figure 2-8A), perhaps because of its regulation by

multiple inputs [9, 32-34]. Importantly, diminished Akt signaling has been noted in TSC-

deficient cells during stimulation with insulin and other growth factors [35-37] . Such

reduction of Akt activity is due to mTORC1-mediated negative feedback that impinges on

multiple targets, including but not limited to IRS1/2 degradation [38, 39] and phosphorylation

and stabilization of GRB10 [40, 41] (Figure 2-7A). While such mTORC1-mediated negative

feedback is well-defined for insulin signaling, little is known regarding its role in the

regulation of cytokine signaling. Interestingly, we found that Tsc1$^{\Delta/\Delta}$ BMDMs display

reduced IRS2 levels in response to IL-4 treatment (Figure 2-7B). This is likely to contribute

to mTORC1-mediated attenuation of Akt signaling, since IRS2 has been implicated in Akt

activation during IL-4 stimulation [29] . Increased levels of phosphorylated and total GRB10

(Figure 2-8B) may also play a role given that GRB10 inhibits signaling downstream of RTKs

[42-44]. Thus increased mTORC1 activity in our model is likely to attenuate Akt signaling at

least in part through IRS2 and GRB10. PDK1, the Akt T308 kinase, has constitutive kinase

activity and is critically regulated by PIP3-mediated recruitment to the plasma membrane [45],

thus normal PDK1 activity in Tsc1$^{\Delta/\Delta}$ BMDMs (Figure 2-8C) also supports our model that

attenuated Akt signaling in Tsc1$^{\Delta/\Delta}$ BMDMs occurs at a receptor proximal step upstream

of PI3K mediated PIP3 production (Figure 2-7A). Finally, LPS-mediated Akt activation was

diminished in Tsc1$^{\Delta/\Delta}$ BMDMs, as evidenced by a defect in phosphorylation of Akt and the

Akt target FOXO1 (Figure 2-8D). Collectively, these findings demonstrate that mTORC1-

mediated negative feedback mechanisms converge to ultimately attenuate Akt signaling in

Tsc1$^{\Delta/\Delta}$ BMDMs.
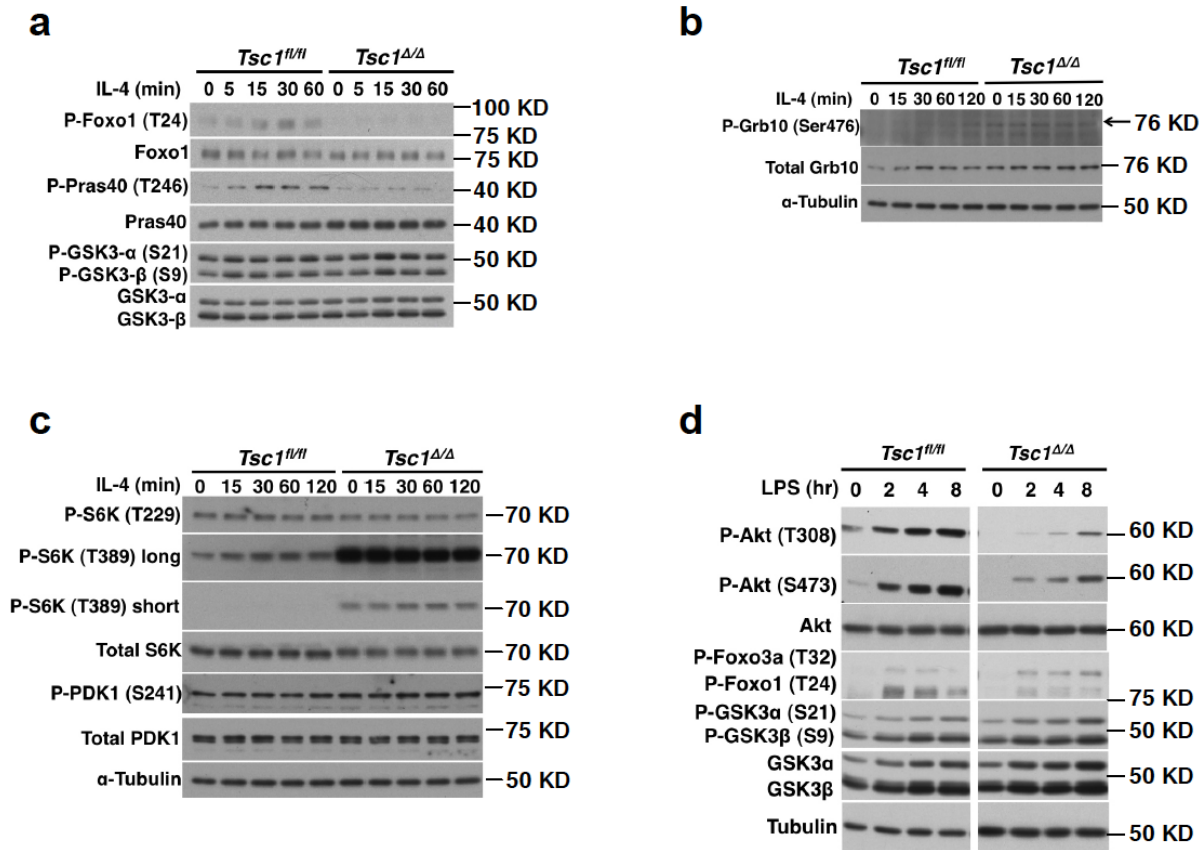
Figure 2-8. IL-4 and LPS-Induced Akt Activation is Attenuated in Tsc1$^{\Delta/\Delta}$ BMDMs

A-C) Immunoblots of Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 as indicated. a. Analysis of Akt targets. b. Elevated levels of total and phosphorylated Grb10 in Tsc1Δ/Δ BMDMs. c. Analysis of phosphorylated and total PDK1. Phosphorylation of PDK1 is not inducible, and levels of phosphorylated and total PDK1 are normal in Tsc1Δ/Δ BMDMs. (AC)

D) Immunoblot analysis of Akt signaling in lysates from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with LPS as indicated. (AC)

Having shown a defect in Akt activation in Tsc1$^{\Delta/\Delta}$ BMDMs, we addressed a potential role in impaired M2 polarization. Previous studies have used rapamycin treatment to alleviate mTORC1-mediated negative feedback of Akt signaling and to interrogate the role of attenuated Akt activation in TSC-deficient models [35-37]. Accordingly, we found that rapamycin treatment of Tsc1$^{\Delta/\Delta}$ BMDMs rescued IL-4 inducible Akt signaling (Figure 2-7C). Importantly, such treatment restored induction of M2 genes Arg1, Fizz1, and Mgl1/2 as well as Arginase activity (Figure 2-7D, E). Together this suggests that deficient M2 polarization in Tsc1$^{\Delta/\Delta}$ BMDMs may be due to mTORC1-mediated negative feedback of Akt signaling. We note that rapamycin treatment of control Tsc1$^{fl/fl}$ BMDMs also modestly increased Akt signaling (Figure 2-7C) as well as M2 responses (Figure 2-7D, E), indicating that acute, signal-dependent activation of mTORC1 during IL-4 signaling can also feedback to inhibit Akt activation.

Attenuated Akt signaling underlies aberrant polarization

Our findings linking reduced Akt activation to impaired M2 polarization in Tsc1$^{\Delta/\Delta}$ BMDMs (Figure 2-7B, C, D, E) are interesting given that little is known regarding the role of Akt in this process. To address this directly, we treated WT BMDMs with MK-2206, an allosteric inhibitor of Akt. This led to a decrease in IL-4-inducible phosphorylation of Akt^{T308} and Akt^{S473} as well as the Akt target FOXO1, but did not affect STAT6 phosphorylation (Figure 2-9A). Importantly, pretreatment with MK-2206 reduced IL-4 mediated induction of Arg1, Fizz1, Mgl2, and Mgl1 (Figure 2-9B), as well as Arginase-1 activity (Figure 2-9C). Similar effects were observed with the structurally distinct Akt inhibitor Aktviii (Figure 2-10A, B), indicating the specificity of the inhibitors. These findings argue that Akt may play an important role in M2 polarization. Together with the data in Figure 3, they also support the idea that attenuated Akt signaling underpins defective M2 polarization in Tsc1$^{\Delta/\Delta}$ BMDMs.

Next, we took a genetic approach to rescue Akt signaling in Tsc1$^{\Delta/\Delta}$ BMDMs. As expected, retroviral transduction of Tsc1$^{\Delta/\Delta}$ BMDMs with myristylated-Akt1 (myr-Akt) led to constitutive Akt signaling as indicated by high basal state P-Akt^{T308} and P-Akt^{S473} (Figure 2-9D). Importantly, this was associated with increased induction of Arg1 and Mgl1 (Figure 2-9E), as well as Arginase-1 activity (Figure 2-9F), following IL-4 stimulation. Although ectopic expression of myr-Akt was insufficient to rescue Fizz1 and Mgl2 expression, this could be achieved in the context of rapamycin co-treatment (Figure 2-10C). Thus, myr-Akt may not fully recapitulate IL-4 inducible Akt activation; or alternatively, rescue of Akt signaling is not sufficient for restoring Mgl2 and Fizz1 expression, and some other consequence of constitutive mTORC1 activation is critically relieved by rapamycin treatment. Finally, to extend these studies to the proinflammatory responses, we examined myr-Akt expressing Tsc1$^{\Delta/\Delta}$ BMDMs following LPS treatment. We observed significantly reduced expression of Il-6 and Tnfα but increased expression of Il-10 (Figure 2-9G and Figure 2-10D).

Deficient M2 polarization in Tsc1$^{\Delta/\Delta}$ mice

Finally, we asked if TSC1-deficiency would impair M2 polarization in vivo. We used intraperitoneal (IP) injection of an IL-4/IL-4 antibody complex to elicit IL-4-dependent recruitment and in situ proliferation of M2 macrophages [46] in Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ mice. Strikingly, induction of most M2 genes was decreased in peritoneal exudate cells (PECs) from Tsc1$^{\Delta/\Delta}$ mice (Figure 2-11A). To corroborate these findings, we used a model of chitin administration that triggers IL-4-dependent recruitment and polarization of M2 macrophages [47, 48]. We observed a near universal reduction of M2 gene expression in Tsc1$^{\Delta/\Delta}$ PECs (Figure 2-11B), similar to the IL-4 injection. Collectively the findings support our model that constitutive mTORC1 activity can attenuate macrophage M2 polarization in vivo.
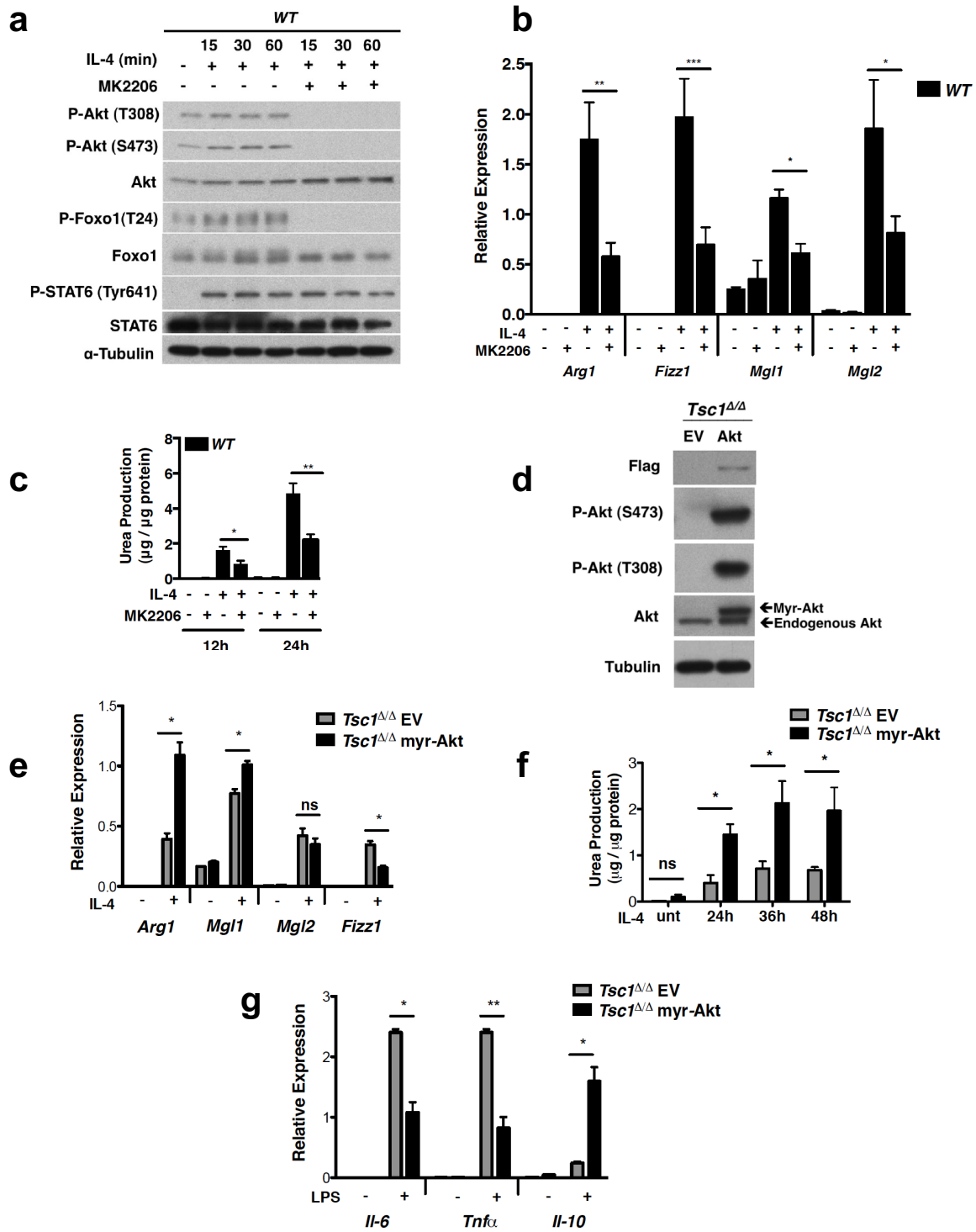
Figure 2-9. Akt signaling is critical for polarization in *Tsc1^{Δ/Δ}* BMDMs.

Figure 2-9 (continued)

A) Immunoblot analysis of WT BMDMs pretreated with MK-2206 for 1 h and treated with IL-4 for the indicated time points. (VB)

B-C) WT BMDMs were pretreated with MK-2206 or DMSO for 1 h before stimulation with IL-4 for 24 h and examination of (**B**) M2 gene expression (n=3) or (**C**) urea production (n=4). (VB)

D) Immunoblot analysis of Tsc1$^{\Delta/\Delta}$ BMDMs transduced with myr-flag-Akt or EV. (AC)

E-F) Myr-Akt Tsc1$^{\Delta/\Delta}$ BMDMs and EV were stimulated with IL-4 followed 24 h later by analysis of (**E**) M2 gene expression (n=4 representative experiments) (**F**) urea production (n=3). (AC)

G) Cytokine gene expression in myr-Akt Tsc1$^{\Delta/\Delta}$ BMDMs and EV Tsc1$^{\Delta/\Delta}$ BMDMs stimulated with LPS for 6 h (n=3). Graphs are shown as mean±s.e.m., *P<0.05, **P<0.01, ***P<0.001. P values were determined using Student's t-tests. (AC)
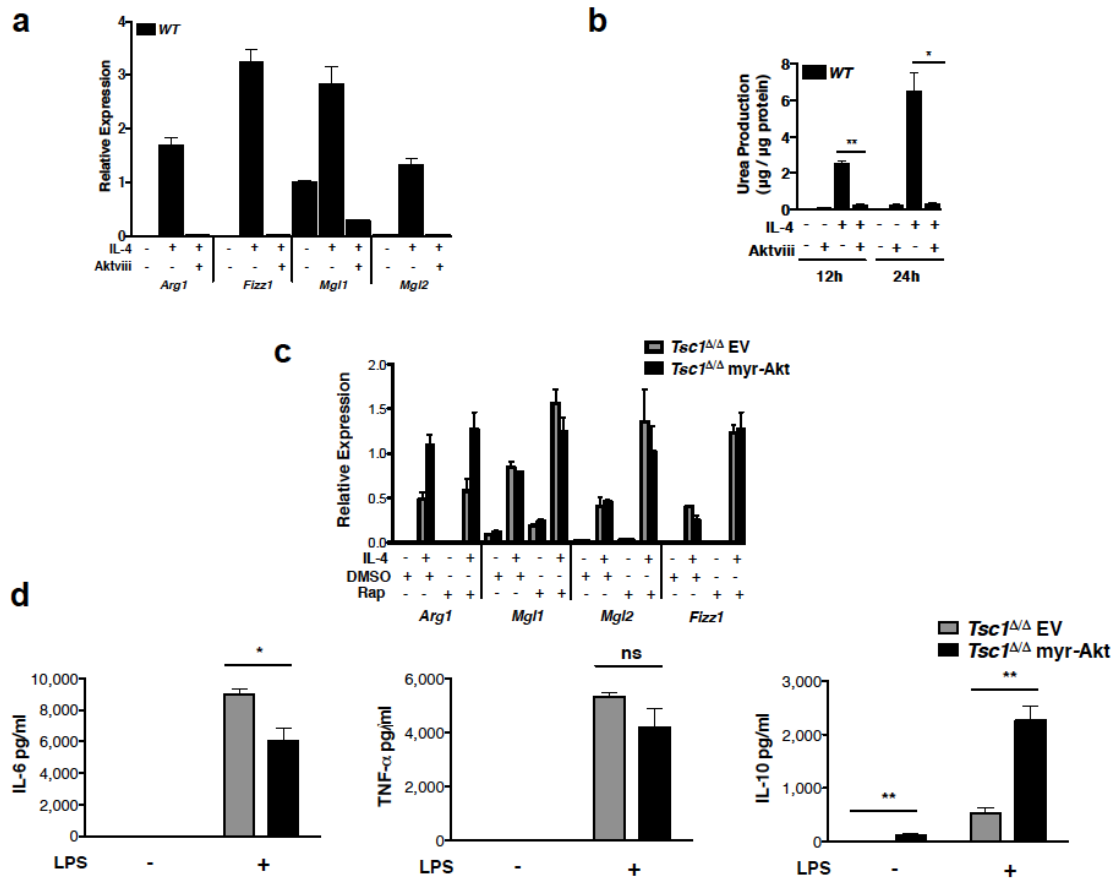
Figure 2-10. Akt Signaling is Critical for Polarization in Tsc1$^{\Delta/\Delta}$ BMDMs

A-B) WT BMDMs were pretreated with Aktviii for 1h before IL-4 stimulation. (A) M2 gene expression was measured after 24h of IL-4 stimulation (representative of 3 independent experiments). (B) Urea production normalized to total protein (n=3). (VB)

C) M2 gene expression in myr-Akt Tsc1$^{\Delta/\Delta}$ BMDMs and EV Tsc1$^{\Delta/\Delta}$ BMDMs pretreated with rapamycin or DMSO for 30min and stimulated with IL-4 for 12h (n=2 representative experiments). (AC)

D) Cytokine concentration in supernatants from myr-Akt Tsc1$^{\Delta/\Delta}$ BMDMs and EV Tsc1$^{\Delta/\Delta}$ BMDMs treated with LPS for 6h (n=3). Graphs are shown as mean ± SEM, *p<0.05, **p<0.01 determined by Student's t-tests. (AC)
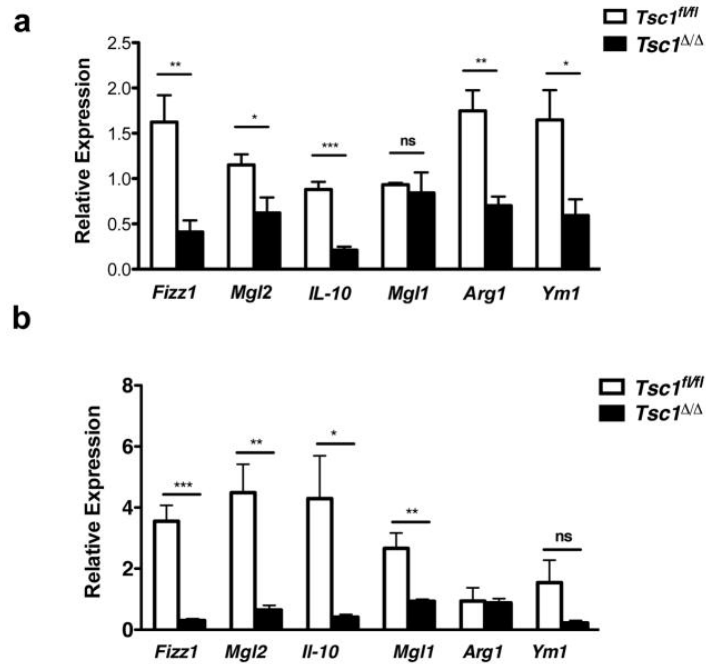
Figure 2-11. M2 Polarization of Tsc1$^{\Delta/\Delta}$ mice is Impaired *In Vivo*

A) M2 gene expression in PECs from Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ mice 4 days post IP injection with IL-4 complex on days 0 and 2 (n= 4 mice per genotype). (AC,VB)

B) M2 gene expression in PECs from male Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ mice 48h post IP injection with chitin (n=5 mice per genotype). Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001. P-values were determined using Student's t-tests.(AC,VB)

Discussion

In this study, we use a novel model of myeloid-specific Tsc1 deletion and constitutive mTORC1 activity to elucidate mTORC1 function in macrophages. We found that $Tsc1^{\Delta/\Delta}$ BMDMs have enhanced proinflammatory cytokine production while IL-10 secretion is reduced, in line with a recent analysis of $Tsc1^{\Delta/\Delta}$ BMDMs [17]. Other studies reached conflicting conclusions, using shRNA knockdown in monocytes and dendritic cells [49, 50]. Extending the analysis of mTORC1 function in macrophages, we showed that $Tsc1^{\Delta/\Delta}$ BMDMs are impaired in M2 polarization, expressing reduced levels of key M2 markers such as Arg-1, Fizz1, Mgl1, Mgl2, Ym1 and Pgc-1β (Figure 2-1D). Interestingly, the defect in M2 activation seems to be selective, since induction of PPARγ and some PPARγ dependent genes (e.g. Cd36 and Fabp4) occur normally (Figure 2-4C, D). Additionally we find that $Tsc1^{\Delta/\Delta}$ BMDMs have diminished levels of Arginase-1 activity and fatty acid oxidation, hallmark features of M2 macrophages (Figure 2-1E, F). This suggests that mTORC1 may couple regulation of fatty acid oxidation to control of macrophage polarization, consistent with the emerging view that macrophage cellular metabolism is closely linked to activation status [3, 8, 51]. Finally, we use models of IL-4 and chitin injection to show that constitutive mTORC1 activity in myeloid lineage cells results in broad defects in M2 polarization in vivo (Figure 2-11).

Normal activation of known regulators of M2 polarization, including STAT6, PPARγ and PPARδ (Figure 2-4), suggests that impaired M2 polarization in $Tsc1^{\Delta/\Delta}$ BMDMs may be due to block of a parallel signaling pathway. In addition to the JAK/STAT6 pathway, IL-4R signaling engages IRS2/PI3K signaling in parallel to mediate Akt activation [29, 30] . While STAT6 is indispensable for M2 polarization [1, 52] , the role of Akt signaling has not been well characterized. Our findings suggest a critical role for mTORC1-mediated feedback inhibition of Akt signaling in $Tsc1^{\Delta/\Delta}$ BMDMs. In support of this, $Tsc1^{\Delta/\Delta}$ BMDMs display decreased IL-4-

inducible Akt activation, as indicated by diminished P-Akt^{T308} and P- Akt^{S473} (Figure 2-7B) and phosphorylation of the Akt targets FOXO1 and PRAS40 (Figure 2-8A). Importantly, rapamycin treatment (Figure 2-7C, D, E) and ectopic expression of myr-Akt (Figure 2-9D, E, F, and Figure 2-10c) restore Akt activation simultaneous with rescue of M2 gene expression and Arginase activity. We suggest that decreased IRS2 (Figure 2-7B) but increased GRB10 (Figure 2-8B) levels may contribute to mTORC1-mediated attenuation of Akt signaling, since IRS2 has been implicated in IL-4R signaling [53] , while GRB10 downregulates signaling downstream of RTKs [42] . While other negative feedback mechanisms have been described in insulin signaling [39, 54] and may exist in our setting, they act synergistically and ultimately converge to attenuate Akt activation. Finally, we believe that feedback inhibition to Akt may also underlie the enhanced responsiveness of Tsc1$^{\Delta/\Delta}$ BMDMs to LPS stimulation. While we have not extensively characterized the underlying mechanism(s) in this context, Akt activation in Tsc1$^{\Delta/\Delta}$ BMDMs is diminished following LPS signaling (Figure 2-8D), and its rescue with myr-Akt expression reduces the exaggerated responses (Figure 2-9G).

Importantly, control of M2 polarization by Akt signaling is likely to extend beyond our genetic model to other settings, since pharmacological inhibition of Akt impairs M2 activation in wild-type BMDMs (Fig. 2-9B, C and Figure 2-10A, B). Moreover, rapamycin treatment of control BMDMs modestly increases Akt signaling and M2 responses (Figure 2-7C, D, E). Thus our findings reveal a largely unappreciated role for Akt in synergizing with the STAT6 pathway to regulate full M2 polarization (Figure 2-12a). Whether Akt promotes or inhibits inflammation downstream of LPS signaling is not entirely clear [55] , but at least in Tsc1$^{\Delta/\Delta}$ BMDMs, simultaneous mTORC1 activation and Akt attenuation lead to enhanced
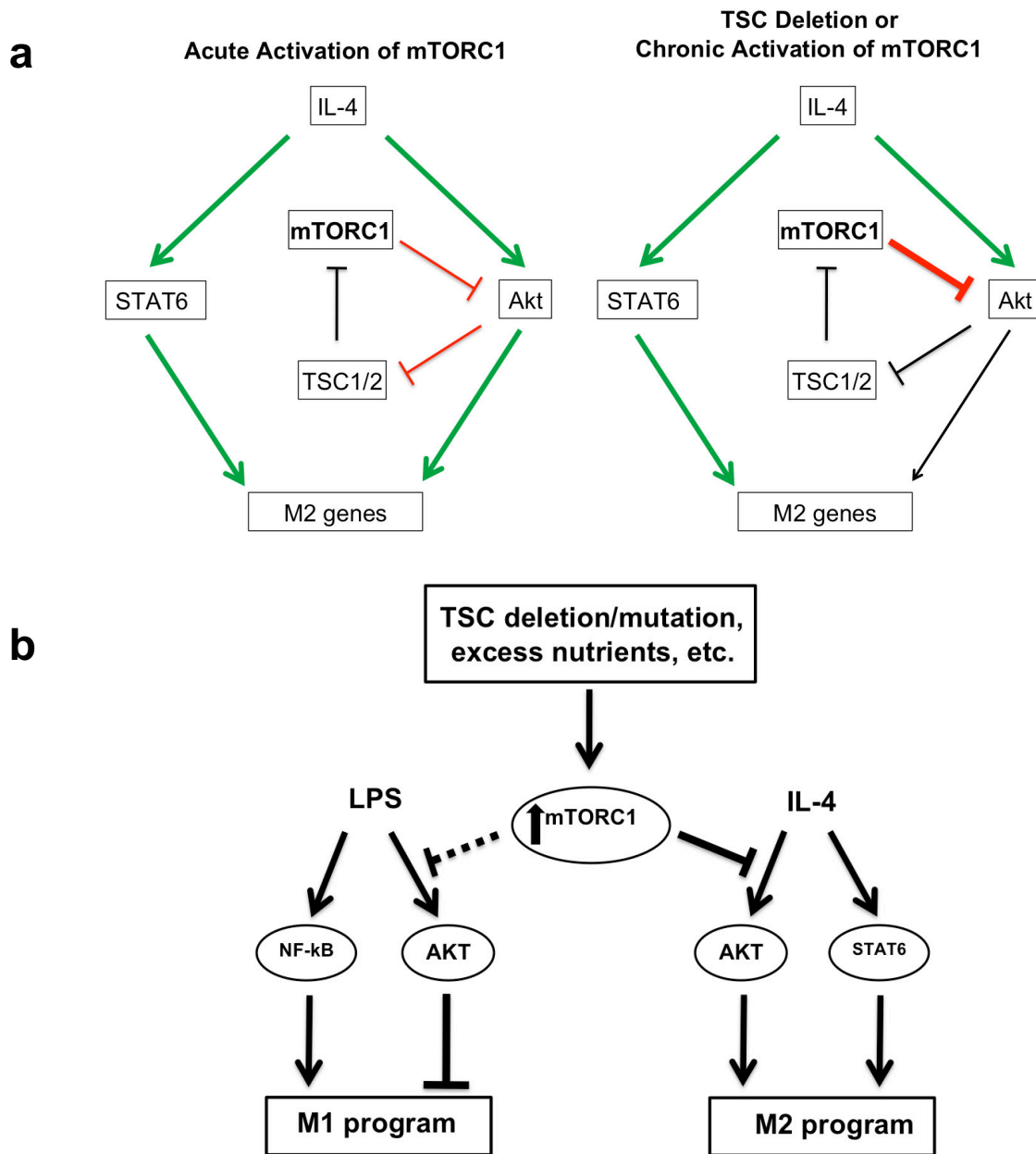
Figure 2-12. Proposed model for how mTORC1 activity controls macrophage polarization.

A) Physiological induction of the Akt-mTORC1 signaling loop by IL-4 stimulation (left) allows

for transient, inducible activation of the pathway, and enables Akt to synergize with the

JAK/STAT pathway for M2 polarization. mTORC1 activity is also regulated by nutrient

availability (not shown here), so such wiring of the signaling pathway may allow calibration of

Figure 2-12 (continued)

M2 activation to metabolic status (left). In contrast, constitutive or aberrant mTORC1

activation corrupts this signaling pathway and modulation of macrophage activation by

metabolic inputs (right). (Green = activation; Red = inhibition; Black = attenuated)

B) Constitutive or aberrant activation of mTORC1 impairs the ability of macrophages to

respond appropriately to polarizing stimuli. A critical mediator of this process is Akt, whose

activity is downregulated by increased mTORC1 activity.

proinflammatory responses that can be rescued by restoring Akt signaling (Figure 2-9G). The relevant Akt targets that regulate macrophage polarization are not well defined, but could include FOXO1 and CEBPβ [56, 57]. Interestingly, macrophages deficient in Rictor, a subunit of the mTORC2 complex that phosphorylates Akt on S473, were deficient in some but not all Akt-dependent activities (Figure 2-13A) consistent with previous models of mTORC2-deficiency [58, 59] but polarized normally to the M2 phenotype (Figure 2-13B,C), and could serve as a plausible model to identify the relevant Akt targets controlling M2 activation.

Our studies indicate the existence of a mTORC1-Akt regulatory loop in the IL-4 signaling pathway that parallels that of the insulin pathway. In the latter, a feedback loop between mTORC1 and Akt—in which receptor engagement of the IRS/PI3K/Akt pathway leads to mTORC1 activation that feeds back to attenuate Akt signaling—is critical for transient, signal-dependent activation of these two signaling modules (Figure 2-7A). Similarly, activation of the IRS2/PI3K/Akt pathway by IL-4 mediates mTORC1 activation (Figure 2-1A, Figure 2-14) and as shown here, results in feedback inhibition of Akt signaling (Figure 2-7A, B, C and Figure 2-12A). Importantly, mTORC1 activity is critically modulated by nutrient/energy availability [60] , thus we propose that integration of the mTORC1-Akt regulatory loop into the IL-4 signaling pathway may allow macrophages to calibrate their activation and function to metabolic status and nutrient availability (Figure 2-12A).

In contrast, this regulatory circuitry is disrupted by constitutive or aberrant activation of mTORC1, as occurs during nutrient excess or in our genetic model. As a consequence, induction of M2 polarization by the synergistic interactions of the Akt and JAK/STAT pathways is impaired (Figure 2-12A, B). Conversely, elevated mTORC1 activity and consequent downregulation of Akt signaling may facilitate increased responses to LPS stimulation (Figure 2-12B). Finally, we note that such "hard-wiring" of mTORC1-Akt signaling to cytokine signaling could have relevance to other immunological contexts, given that many cytokines that regulate
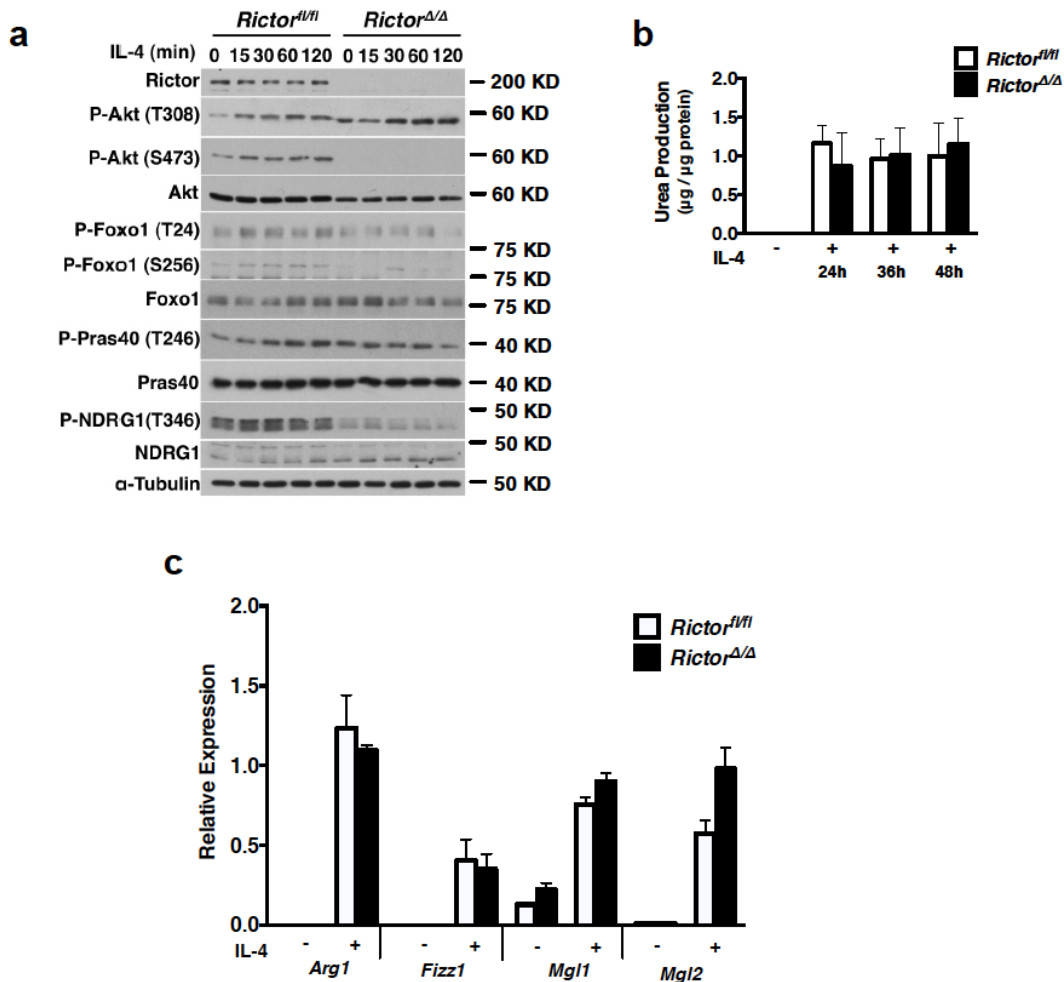
Figure 2-13. Intact M2 Polarization in Rictor$^{\Delta/\Delta}$ BMDMs

A) Reduced phosphorylation of the Akt target Foxo1, but not Pras40 in Rictor$^{\Delta/\Delta}$ BMDMs. Immunoblot analysis of lysates from Rictor$^{fl/fl}$ and Rictor$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 as indicated. (VB)

B) Urea production normalized to total protein after stimulation with IL-4 as indicated (n=2 representative experiments). (VB, AC)

C) M2 gene expression analysis in Rictor$^{fl/fl}$ and Rictor$^{\Delta/\Delta}$ BMDMs stimulated with IL-4 for 24h (n=2 representative experiments). Graphs are shown as mean ± SEM. (VB, AC)
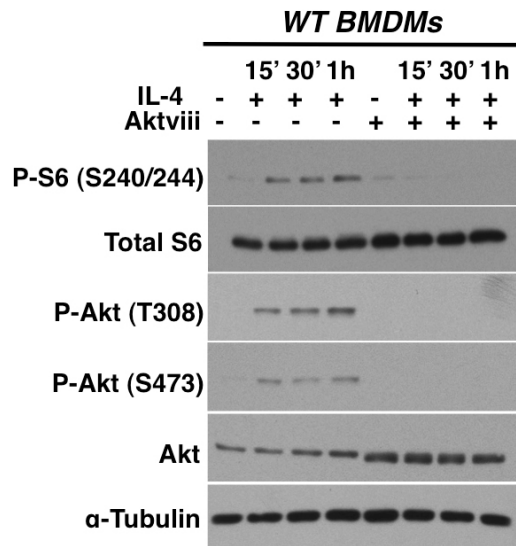
Figure 2-14. Akt Signaling is Critical for mTORC1 Activation in BMDMs.

Immunoblot analysis of WT BMDMs pretreated with Aktviii for 1h before IL-4 stimulation for the indicated times. Reduced phosphorylation of S6 on S240/244 indicates attenuated mTORC1 activity in the presence of Aktviii. (VB)

immune cell function engage the Akt pathway (and presumably also mTORC1).

In conclusion, our study highlights a key role of the mTORC1 pathway in control of macrophage polarization. Such control is likely to be of particular relevance for adipose tissue macrophage (ATMs). In the lean state, ATMs with a M2 phenotype maintain an anti-inflammatory environment and adipocyte insulin sensitivity, while in obesity, ATMs with a M1 phenotype produce inflammatory cytokines and promote insulin resistance and metabolic dysfunction. We propose that nutrient sensing by mTORC1 may directly regulate the pathophysiological switch of ATMs during obesity, extending the current model for regulation of the pathophysiological M2 to M1 switch [61] . Indeed, given that macrophages are critical orchestrators of diverse physiological responses, regulation of macrophage activation by the mTOR pathway may have profound consequences in many settings, including helminth infection, inflammation, allergy, and tissue repair.

Materials and Methods

BMDMs

Briefly, femurs were removed from mice after CO_2 euthanasia, and cells were liberated using a mortar and pestle. For macrophage differentiation, bone marrow derived cells were plated in petri dishes with 1640 RPMI media (10% FCS, Penicillin/Streptomycin, 2mM L-Glutamine) supplemented with MCSF-containing L929 cell supernatant for seven days. MCSF differentiated macrophages were harvested and plated in tissue culture dishes for subsequent experiments. For M1-like activation, $0.5\text{-}0.7\text{x}10^6$ BMDMs were plated in 12-well tissue culture dishes and treated with 10ng/ml LPS (Invivogen). For M2(a) polarization, cells were treated with 10ng/ml IL-4 (Peprotech). For M2b polarization, cells were treated with 10ng/ml IL-10 (Peprotech). For M2b polarization, cells were treated simultaneously with 10ng/ml LPS (Invivogen) and either un-opsonized sheep red blood cells (SRBC) (Lampire biological

laboratories) or SRBCs opsonized with 1:400 anti-SRBC IgG (cat# 55806 MP Biomedicals).

Mice

To generate mice with targeted deletion of Tsc1 in myeloid lineage cells, mice with flanking

loxP Tsc1 alleles (Tsc1$^{fl/fl}$) were crossed to LysozymeM-Cre transgenic mice, both on a B6

background [37, 62]. Cre-recombinase activity results in deletion of exons 17 and 18 of Tsc1,

generating a null allele [63] . Male mice age 12 weeks were used for in vivo chitin

administration and male mice age 6-8 weeks were used for in vivo IL-4 administration. For

Rictor deletion in vivo, tamoxifen (VWR) was suspended in sunflower seed oil (VWR) at

10mg/ml, and 200μl/25g body weight was injected into 10-week old Rictor$^{fl/fl}$ and Rictor$^{fl/fl}$

UbiquitinC- CreERT2 mice once daily for 7 days [64] . Bone-marrow from such mice were

used to generate Rictor-deficient macrophages . Mice were maintained at Harvard Medical

School and Massachusetts Institute of Technology, and all procedures were performed in

accordance with the guidelines set forth by the Institutional Animal Care and Use Committees

at each institution.

Immunoblotting

For protein sample preparation, cells were washed twice with cold PBS following stimulation

and lysed in 1% NP-40 buffer with EDTA-free protease inhibitor tablets (Roche Diagnostics)

and phosphatase inhibitors. Protein concentration in lysates was determined using the

Bradford method. Equal amounts of protein were loaded onto SDS-PAGE gels and

subsequently transferred to PVDF membranes for Immunoblotting with primary antibodies as

indicated.

Antibodies and Reagents

Primary antibodies were purchased from Cell Signaling (all at 1:1000 dilution in 5% BSA), except for the following: α- Tubulin (Sigma, 1:5000), β-Actin (Sigma,1:2000), Flag-M2 (Sigma, 1:1000), and PPARγ (Santa Cruz E-8, 1:1000) and PPARδ (Santa Cruz, 1:250). For flow cytometry, antibodies were used to CD11b-PE (BD Biosciences) and F4/80-FITC (BioLegend). Inhibitors were used as follows: MK2206 1µM (Selleck), Aktviii 10µM (Enzo), Rapamycin 20nM (LC Laboratories), Troglitazone 1µM (Cayman) and GW501516 100 µM (Enzo).

Arginase Assays

Arginase assay was described previously [65] . Briefly, 0.5×10^6 cells/well in 12-well plates were stimulated with IL-4 for 12-48h. Cells were lysed in 0.1% TritonX-100 lysis buffer with protease inhibitors. Lysates with equal amounts of protein were incubated with 500mM L-Arginine for 45 minutes at 37°C, followed by acid stop solution. The degradation of L-arginine to urea was measured by adding 9% isonitrosopropiophenone in 100% ethanol. Absorbance was read at 540nm in a microplate reader. All samples were read in triplicate.

Fatty Acid Oxidation

BMDMs were plated 0.7×10^6 cells/well in 12-well tissue culture dishes in complete RPMI and stimulated with IL-4 for 36h. After stimulation, cells were washed with PBS and loaded with low glucose DMEM + 2% fatty acid-free BSA (Lampire Biologicals) for 30 minutes at 37°C. After 30 minutes, cells were washed with PBS and given ^{3}H-labeled palitic acid (2µCi/well, MP Biomedicals) in low glucose DMEM, with 2% fatty acid-free BSA and 0.2mM unlabeled oleic acid (Sigma). After 4h, 100µl of media was collected and the isolation of ^{3}H$_2$O was performed using trichloroacetic acid followed by chloroform-methanol extraction. The water-soluble fraction was collected in 5ml of EcoLume (MP Biomedicals) scintillation fluid and counted for 5

minutes using a Beckman LS6500 scintillation counter. Cells were lysed in 500µl of 0.1N NaOH

and total protein was determined using the Bradford method. Background ^{3}H was subtracted

from the CPM (counts per minute) value and all samples were normalized to mg of total protein.

All samples were performed in duplicate.

Myr-Akt-Transduction

To make retrovirus particles, 293T cells were co-transfected with pBabe empty vector

(EV) Puro or pBabe Puro Myr Flag Human-Akt1 purchased from Addgene (plasmid

15294) along with pCL-Eco (Imgenex #10045P) using Lipofectamine 2000 (Invitrogen).

Transfected 293T media was changed the next day and placed at 32C. Viral supernatant was

collected on day 2 and day 3 post-transfection. Fresh bone marrow was plated on the same

day as transfection above and transduced with media containing viral supernatant (50% viral

supernatant containing pBabe EV or pBabe Puro Myr Flag Human-Akt1, 40% RPMI complete

media, and 10% CMG-media) on day 2 and day 3 and selected using 4 µg/ml puromycin on

days 4-7.

ELISA

Cytokine concentration was determined using for IL-10, TNFα, and IL-6 using ELISA kits

purchased from BioLegend. Briefly, experimental supernatants were collected and centrifuged

at 3000 g/5min. Supernatants were analyzed in duplicate per manufacturers protocol.

Chromatin Immunoprecipitation

For STAT6 ChIP, BMDMS ($5x10^6$) were plated in 6cm tissue culture plates and stimulated for

2h with 10ng/ml IL-4. Cells were subsequently fixed with 1% formaldehyde for 10 min at room

temperature. Formaldehyde was quenched with glycine. After collecting cells, lysis was

performed using 500µl of SDS buffer (1% SDS, 10mM EDTA, 50mM Tris-Cl pH 8) plus

protease and phosphatase inhibitors. Cells were subsequently sonicated for 3 min on ice with

1 sec pulses using a Misonix 4000 sonicator to shear chromatin. Following sonication,

samples were diluted to 3ml with ChIP Dilution Buffer (0.01% SDS, 1.1% TritonX-100, 1.2mM

EDTA, 16.7mM Tris-Cl pH 8, 167mM NaCl) and precleared for 1h at 4°C with Protein A

salmon sperm/agarose beads (Millipore). Precleared chromatin was split into 400µl aliquots

for IP with either 5µg of STAT-6 (M-20 ChIP grade, Santa Cruz) or for no antibody control

overnight at 4°C. IPs were incubated with Protein A salmon sperm/agarose beads (Millipore)

the following day for 3h at 4°C. After 3h, beads were spun down and ~10% of chromatin was

taken for input and processed in parallel to IP samples. Antibody-bead complexes were then

washed with low salt (0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH 8, 150mM

NaCl), high salt (0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-Cl pH 8, 500mM NaCl),

and lithium chloride (0.25M LiCl, 1% deoxycholic acid, 1%NP-40, 1mM EDTA, 10mM Tris-Cl

pH 8) buffers followed by two washes with TE buffer. Chromatin-antibody complexes were

eluted with elution buffer (1%SDS + 0.1M sodium bicarbonate) and crosslinks were reversed

using sodium chloride and incubation at 60°C for 4h. Samples were then incubated 1h with

Proteinase K (Roche) at 60°C. DNA was purified using PCR purification columns (Qiagen) and

used for quantitative PCR with primers generated to the Arg1 promoter region. Fold

enrichment was calculated as ChIP signals divided by no antibody control and normalized to

input.

Quantitative PCR

To measure gene expression in BMDMs and PECs, RNA was isolated using RNA Bee (Tel-

Test) per manufacturers protocol and used to make cDNA using High Capacity cDNA Reverse

Transcription Kit (Applied Biosystems). A Bio-Rad C1000 Thermocycler was used to analyze the

samples under the following conditions: 95°C (5 min), 50 cycles of 95°C (10 s), 60°C (10 s), and

72°C (20 s). Reaction mixture consisted of 4 µl cDNA, 1.5 µl 3 µM primers for each gene used in

the study (F+R), 2 µl H_2O, and 7.5 µl 2x SYBR green (Bio-Rad). Samples from BMDMs were

normalized to hypoxanthine phosphoribosyltransferase (HPRT) and samples from PECs were

normalized to the macrophage marker CD68. Data was analyzed by means of the CFX Manger

Software (Bio-Rad) using the delta/delta CT method.

Chitin Administration

Chitin (Sigma) was prepared as previously described [47] . Briefly, chitin was washed 3 times

with PBS and then sonicated (Misonix Sonicator 4000) for 30 minutes on ice. The dissolved

chitin was filtered and diluted with PBS to 4 µg/ml. 800ng chitin dissolved in 200 µl PBS of was

injected intraperitoneally and PECs were collected 48 hours post injection.

IL-4 Complex Administration

Long acting IL-4 complex (IL-4c) was prepared as previously described [46] . Briefly, IL-4

(Peprotech) was suspended at a concentration of 500 µg/ml and mixed with anti-mouse IL-4

(BioXcell clone 11b11) at a molar ratio of 2:1 (weight 1:5) and incubated 1-2 minutes at room

temperature. IL-4c was suspended in normal saline to a concentration of 25µg/ml IL-4 and 125

ug/ml of 11b11. Each mouse was injected intraperitoneally with 200µl of IL-4c (5 µg IL-4 and 25

µg 11b11) on day 0 and 2, and PECs were collected on day 4.

Dual Luciferase Assay

BMDMs were electroporated using Amaxa nucleofector and mouse macrophage nucleofector

kit (Lonza) with PPAR-Firefly luciferase plasmid (C.H. Lee, Harvard School of Public Health) or

STAT6-Firefly luciferase (purchased from Addgene-plasmid #35554) along with Renilla-

Luciferase plasmid as a transfection control. The PPAR- Firefly luciferase plasmid consists of 3 copies of the Acox1 PPAR response element [66] upstream of an SV40 minimal promoter. BMDMs were treated with IL-4 and/or troglitazone 4 hours post electroporation for 24 hours. Cell lysates were collected and analyzed using the Promega Dual-Luciferase Reporter Assay System.

Statistical Analysis

Statistical analysis was carried out using Prism (Graph Pad) software. The student's t-test was used to determine statistical significance, defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

Acknowledgements:

Author contributions

V.B. and A.J.C designed and performed experiments, analyzed data, and wrote the paper. T.H. supervised the project, including experimental design and data analysis, and edited the paper. I.B.-H. and D. W. L. contributed technical expertise. D.M.S. and B.D.M. provided reagents and mice.

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CHAPTER 3

The Akt-Acly pathway is a critical regulator of M2 polarization

Adapted from:

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Abstract

Macrophage activation/polarization to distinct functional states is critically supported by metabolic shifts. How polarizing signals coordinate metabolic and functional reprogramming, and the potential implications for control of macrophage activation, remains poorly understood. Here we show that IL-4 signaling co-opts the Akt-mTORC1 pathway to regulate Acly, a key enzyme in Ac-CoA synthesis, leading to increased histone acetylation and M2 gene induction. Only a subset of M2 genes is controlled in this way, including those regulating cellular proliferation and chemokine production. Moreover, metabolic signals impinge on the Akt-mTORC1 axis for such epigenetic control of M2 activation. We propose that Akt-mTORC1 signaling calibrates metabolic state to energetically demanding aspects of M2 activation, which may define a new role for metabolism in supporting macrophage activation.

Introduction

Macrophages are pleiotropic cells that assume a variety of functions depending on tissue of residence and tissue state. Their ability to acquire diverse, context-dependent activities requires activation (or polarization) to distinct functional states, triggered by a variety of factors including microbial products, cytokines, and growth factors(1, 2). M1 or classical activation is triggered during infection by microbial products including LPS, leading to the upregulation of a transcriptional program encoding antimicrobial activities and inflammatory cytokines. M2 or alternative activation is triggered by IL-4 and IL-13 produced during parasite infections, and activates the transcription factor Stat6 to induce a transcriptional program that encodes pro-fibrotic and tissue remodeling activities and Type 2 inflammation(1, 2).

While macrophage activation is relatively well-understood at the level of signal transduction, transcriptional regulation, and acquisition of new effector activities, the metabolic underpinnings remain less clear. An emerging view is that macrophage activation to particular states is associated with distinct metabolic shifts(3-5). For example, M1 macrophages upregulate glucose and glutamine utilization(6, 7), while M2 macrophages augment β-oxidation and glutamine consumption(8, 9). Importantly, such metabolic shifts critically support macrophage activation. Increased glycolytic flux in M1 macrophages is coupled to de novo lipogenesis, which enables ER and Golgi expansion and production of high levels of inflammatory cytokines(10). Another consequence of enhanced glycolysis is accumulation of the TCA cycle metabolite succinate, leading to stabilization of the transcription factor HIF-1α and transcriptional induction of *Il1β* and other target genes in the M1 macrophage(6). How oxidative metabolism boosts M2 activation is not clear, but glutamine metabolism fuels production of UDP-GlcNAC, an important modification of multiple M2 markers(9).

Consistent with the idea that macrophage activation is supported by metabolic shifts, recent studies indicate that macrophage polarizing signals impinge on metabolic signaling

pathways. Polarizing signals like LPS and IL-4 regulate the activity of Akt, mTORC1, and AMPK(10-13), presumably to coordinate metabolic processes that critically underlie macrophage polarization. Limited studies indicate that perturbing the activity of these metabolic regulators impairs macrophage metabolism and activation(10, 12). For example, Akt mediates enhanced glycolysis to support lipid synthesis and inflammatory cytokine secretion in M1 macrophages(10). Akt similarly stimulates glucose-fueled lipid synthesis in growing and proliferating cells, where lipids are used to build cellular membranes(14). Therefore, M1 macrophages co-opt a metabolic process (Akt-dependent lipogenesis) in order to coordinate a macrophage-specific function (inflammatory cytokine secretion). In general, however, how polarizing signals control metabolic shifts, and the full implications of this for control of macrophage activation, remains poorly understood.

Here we show that integration of the Akt-mTORC1 pathway into IL-4 signaling allows for selective control of some M2 responses. Control is exerted at the level of Acly, a key enzyme in Ac-CoA production, thus modulating histone acetylation and transcriptional induction of a subset of M2 genes. Consistent with its role as a key metabolic sensor, the Akt-mTORC1 pathway couples metabolic input to gene-specific control of M2 gene induction. These findings reveal subsets of the M2 response, including chemokine production and cellular proliferation, that are linked to metabolic state by Akt-mTORC1 signaling.

Results

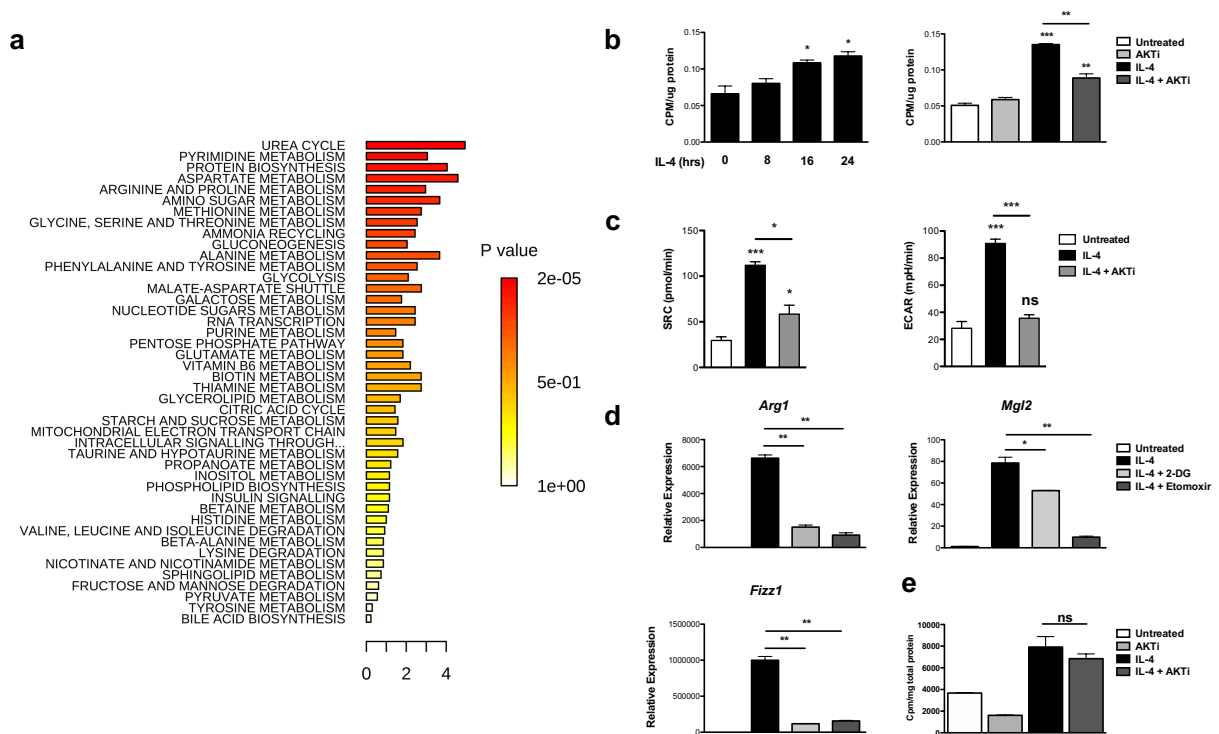***Akt regulates increased glucose metabolism in M2 macrophages***

Akt is a major metabolic regulator implicated in M2 activation(11, 15), but the underlying mechanisms remain poorly characterized. To begin to address this question, we employed unbiased metabolic profiling of M2 macrophages, using LC/MS-based metabolomics and a platform that measures ~290 small metabolites representative of all major pathways of

intermediary metabolism(16). Top enriched pathways include urea cycle and arginine and proline metabolism, consistent with previous studies indicating upregulation of arginine metabolism in M2 macrophages(17), as well as amino acid utilization and metabolism and nucleotide metabolism (Figure 3-1A). Other top enriched pathways include glycolysis, amino sugar metabolism, and glycine, serine, and threonine metabolism, suggesting altered flux through glycolysis and glycolytic shunts (Figure 3-1A).

As M2 activation is thought to be sustained by fatty acid rather than glucose utilization (7, 8), we decided to re-examine the role of glycolysis in M2 macrophages. We found that BMDMs increased glucose uptake in a time-dependent manner in response to IL-4 treatment. Such increase was reduced by cotreatment with the Akt inhibitor MK2206 (Figure 3-1B), indicating control by Akt and consistent with a role for Akt in regulating glycolysis in many settings(14). Moreover, enhanced glucose consumption in M2 macrophages was associated with an Akt-dependent increase in both glycolysis and oxidative metabolism, as indicated by extracellular flux assays (Figure 3-1C). Importantly, glycolytic flux was needed for optimal implementation of the M2 program. Similar to the β-oxidation inhibitor etomoxir, the glycolysis inhibitor 2-DG reduced IL-4-mediated induction of some M2 genes (Figure 3-1D). Therefore, Akt mediates enhanced glucose consumption in M2 macrophages, and this contributes to induction of M2 gene expression. Such glucose consumption may also fuel production of UDP-Glc-NAc, the substrate for glycosylation of some M2 markers(9). In contrast, Akt does not control β-oxidation in M2 macrophages (Figure 3-1E).

IL-4 signaling activates Akt to allow for selective control of M2 gene induction

Because the increase in glucose utilization was relatively modest, we considered that Akt could play additional roles in control of M2 activation and turned to an analysis of M2 gene regulation. We examined induction of Fizz1 (also called Retnla or Relma), Arg1, Mgl2, Ym1

Figure 3-1. Akt regulates enhanced glucose utilization in M2 macrophages

A) Top metabolic pathways enriched in M2 macrophages as identified by LC/MS-based metabolomics profiling. (AC, IBS)

B) M2 macrophages increase glucose uptake in an Akt-dependent manner. BMDMs were treated with IL-4 for the indicated time periods (left) or 16 h +/- the Akt inhibitor MK2206 (Akti) (right), followed by analysis of uptake of ^{3}H-deoxy-D-glucose. (JY)

C) Increased glucose utilization in M2 macrophages is associated with enhanced oxidative metabolism and glycolysis. BMDMs were treated with IL-4 for 20 h +/- Akt inhibitor, followed by analysis of spare respiratory capacity (SRC) and aerobic glycolysis (ECAR) in extracellular flux analyses. (IA)

D) M2 gene induction is sensitive to the glycolysis inhibitor 2-deoxyglucose (2-DG). BMDMs were treated with IL-4 +/- 2-DG or the β-oxidation inhibitor etomoxir, followed by analysis of M2 gene induction by qPCR. (AC, JY)

Figure 3-1 (continued)

E) Akt does not regulate β-oxidation in M2 macrophages. BMDMs stimulated with IL-4 +/- Akt inhibitor were incubated for 3 h with ^{3}H-palmitate for analysis of β-oxidation. (VB)

The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.

Cd36, and Fabp4, "hallmark" M2 genes commonly used in studies of M2 activation(17).

Consistent with the role of Stat6 as a transcriptional master regulator of M2 activation(18),

induction of these M2 genes was ablated in Stat6 KO BMDMs (Figure 3-2A). Importantly and

as reported(11, 15), Akt activity controlled the induction of a subset of M2 genes. In the

presence of the Akt inhibitor MK2206, induction of Arg1, Fizz1, and Mgl2 was reduced ~40-80%,

while Ym1, Cd36, and Fabp4 were not affected (or even super-inducible) (Figure 3-3A). Use of

a structurally distinct Akt inhibitor, Aktviii, yielded similar results, suggesting specificity in

inhibition (data not shown). Below, these two groups of genes will be referred to as Akt-

dependent and Akt-independent M2 genes, respectively.

The IL-4R activates Jak-Stat signaling as well as Akt-mTORC1 signaling in

macrophages (11) (Figure 3-2B). Receptor ligation activates the latent activity of Jak1 and Jak3

kinases, leading to phosphorylation and activation of Stat6, as well as engagement of the

adaptor protein IRS2. IRS2 recruits PI3K, which generates PIP3 from PIP2 leading to

phosphorylation and activation of Akt. Activated Akt phosphorylates and inactivates the TSC

complex, a negative regulator of mTORC1, to activate mTORC1. While the precise relationship

between Jak-Stat and Akt-mTORC1 signaling remains unclear, the data in Fig 2A and Figure 2-

figure supplement 1 suggests that they may operate in parallel and independently downstream

of the IL-4R. Indeed, IL-4-mediated increases in Stat6 activation, as indicated by

phosphorylation on Y641, was not affected in the presence of an Akt inhibitor (Figure 3-3B).

Stat6 activity as measured by a Stat6-dependent luciferase reporter was also not impaired by

inhibition of Akt activity (Figure 3-2C). Conversely, WT and Stat6 KO BMDMs could similarly

activate Akt, as indicated by phosphorylation on S473, as well as mTORC1, as indicated by

phosphorylation of the mTORC1 target S6K, in response to IL-4 (Figure 3-3B). These findings

support the idea that the Jak-Stat and Akt-mTORC1 pathways are independent signaling
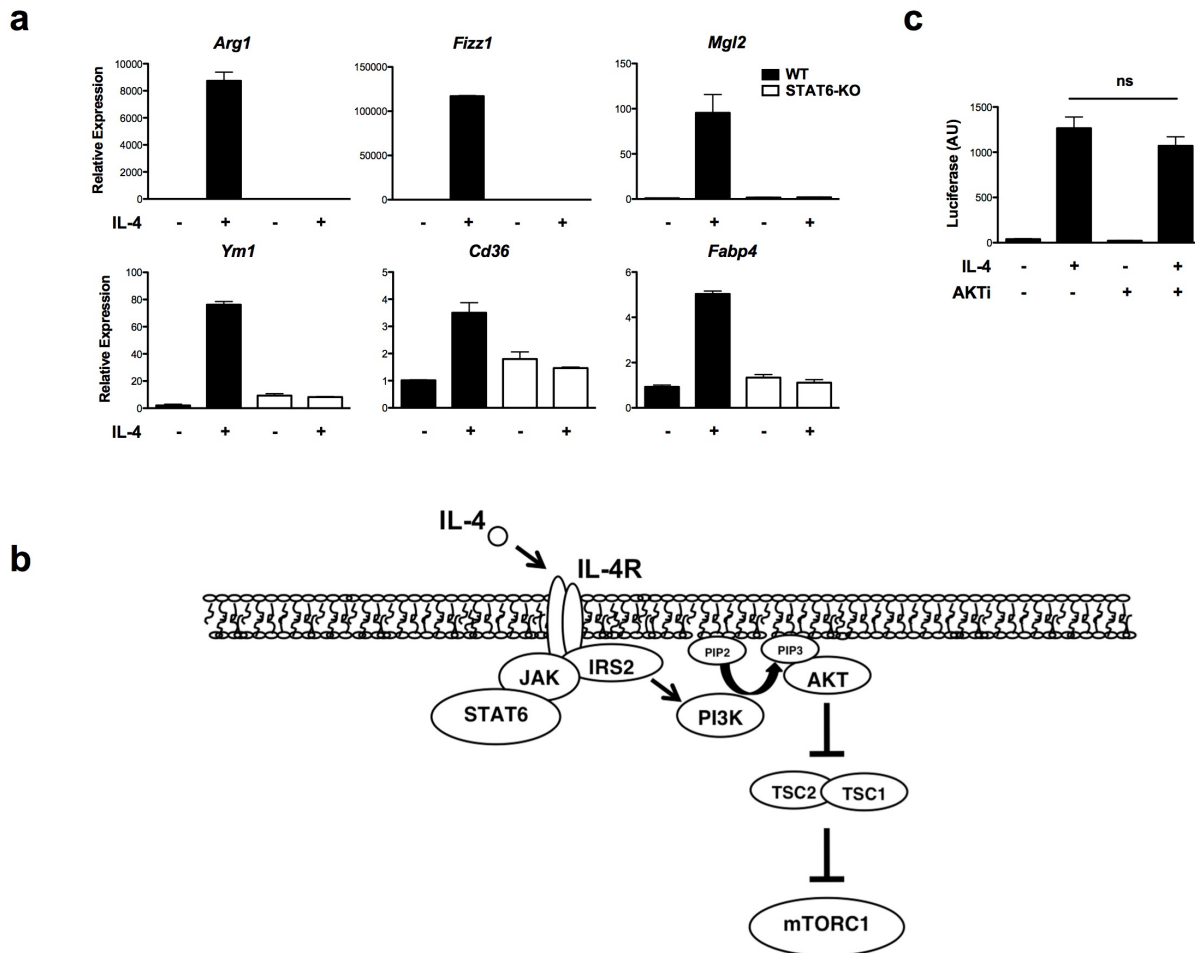
Figure 3-2: Stat6 and Akt-mTORC1 pathways are independent signaling branches downstream of the IL-4R

A) M2 gene induction is dependent on Stat6. WT and Stat6 KO BMDMs were stimulated with IL-4 followed by analysis of M2 gene induction by qPCR. (AC)

B) IL-4 signaling activates the Jak-Stat and Akt-mTORC1 pathways. Receptor ligation activates the latent activity of Jak1 and Jak3 kinases, leading to phosphorylation and activation of Stat6. Jak-mediated phosphorylation of the IL-4R also allows engagement of the adaptor protein IRS2. IRS2 recruits PI3K, which generates PIP3 from PIP2 at the plasma membrane, followed by recruitment, phosphorylation, and activation of Akt. Activated Akt phosphorylates and inactivates the TSC complex, a negative regulator of

Figure 3-2 (continued)

mTORC1, to activate mTORC1. (AC)

C) Stat6 transcriptional activity is not affected by block of Akt activity. BMDMs were

transfected with a Stat6-dependent luciferase reporter, followed by IL-4 stimulation +/-

Akt inhibitor. Stat6 activity was assessed by a luciferase assay. (AC)

The student's t-test was used to determine statistical significance, defined as *P<0.05,

**P<0.01, and ***P<0.001.
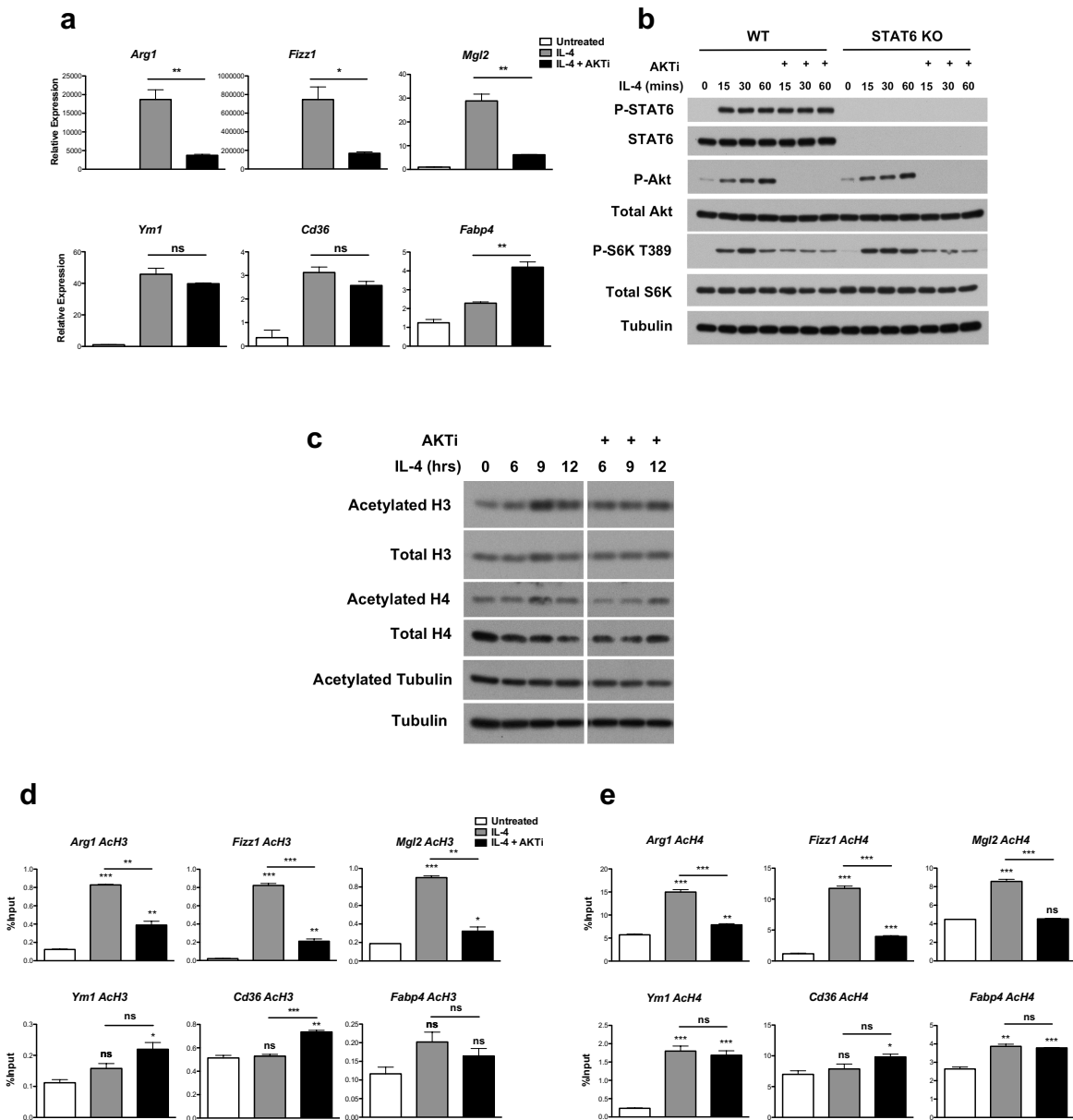
Figure 3-3: Akt regulates inducible histone acetylation at some M2 genes

A) Akt activity stimulates induction of a subset of M2 genes. BMDMs were stimulated with IL-4 +/- the Akt inhibitor MK2206 (Akti), followed by analysis of M2 gene induction by qPCR. (AC)

B) The Jak-Stat and Akt-mTORC1 pathways are activated independently downstream of the IL-4R. WT and Stat6 KO BMDMs were stimulated with IL-4 +/- Akt inhibitor as indicated. Analysis of Stat6, Akt, and mTORC1 activation was assessed by western blotting. (AC)

Figure 3-3 (continued)

C) IL-4 increases global histone acetylation in an Akt-dependent manner. BMDMs stimulated as indicated were analyzed by western blotting for H3 and H4 acetylation. (AC)

D-E) Akt regulates inducible H3 (D) and H4 (E) acetylation at some M2 genes. BMDMs stimulated as indicated were subject to ChIP analysis using antibodies to acetylated H3 or acetylated H4. Enrichment of the indicated M2 gene promoters was assessed by qRT-PCR. (AC)

The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.

branches downstream of the IL-4R, and suggest a basis by which all M2 genes are controlled by Stat6 while a subset receives additional inputs from the Akt-mTORC1 pathway.

A seminal study from Wellen and colleagues indicated that Akt signaling in cancer cells and differentiating adipocytes links metabolic state to gene expression via effects on histone acetylation(19), thus we hypothesized that Akt-mTORC1 signaling may control histone acetylation to regulate M2 gene expression. Indeed, IL-4-treatment of BMDMs enhanced global acetylation of H3 histones, as indicated by western blot of whole cell lysates (Figure 3-4A). Importantly, IL-4-inducible increases in global H3 and H4 acetylation were reduced by cotreatment with an Akt inhibitor, indicating at least partial dependence on Akt (Figure 3-3C and 3-4B). In contrast, tubulin acetylation was not modulated by IL-4 treatment (Figure 3-4B). We next examined gene-specific patterns of H3 and H4 acetylation by chromatin immunoprecipitation (ChIP) experiments. IL-4 treatment increased H3 and H4 acetylation at promoters of M2 genes (Figure 3-3D, E), with the degree of inducible acetylation correlating well with the degree of gene induction (Figure 3-3A) (despite some small variability such as greater increases in H4 versus H3 acetylation at the Ym1 promoter). Interestingly, such increases in H3 and H4 acetylation were reduced by an Akt inhibitor at M2 genes induced in an Akt-dependent manner (Arg1, Fizz1, Mgl2), but not at M2 genes induced independently of Akt (Ym1, Cd36, Fabp4) (Figure 3-3D, E). Pol II recruitment to M2 gene promoters paralleled H3 and H4 acetylation, and was controlled by Akt at M2 genes induced in an Akt-dependent manner (Figure 3-4). Together, these findings support the hypothesis that Akt regulates histone acetylation and Pol II recruitment at a subset of M2 genes.

Akt-mTORC1 signaling regulates Acly to control Ac-CoA production in M2 macrophages

How might Akt regulate increased histone acetylation in M2 macrophages? We hypothesized that Akt may control production of Ac-CoA, the metabolic substrate for histone acetylation. Using quantitative stable isotope dilution-LC-MS, we found that IL-4 treatment led
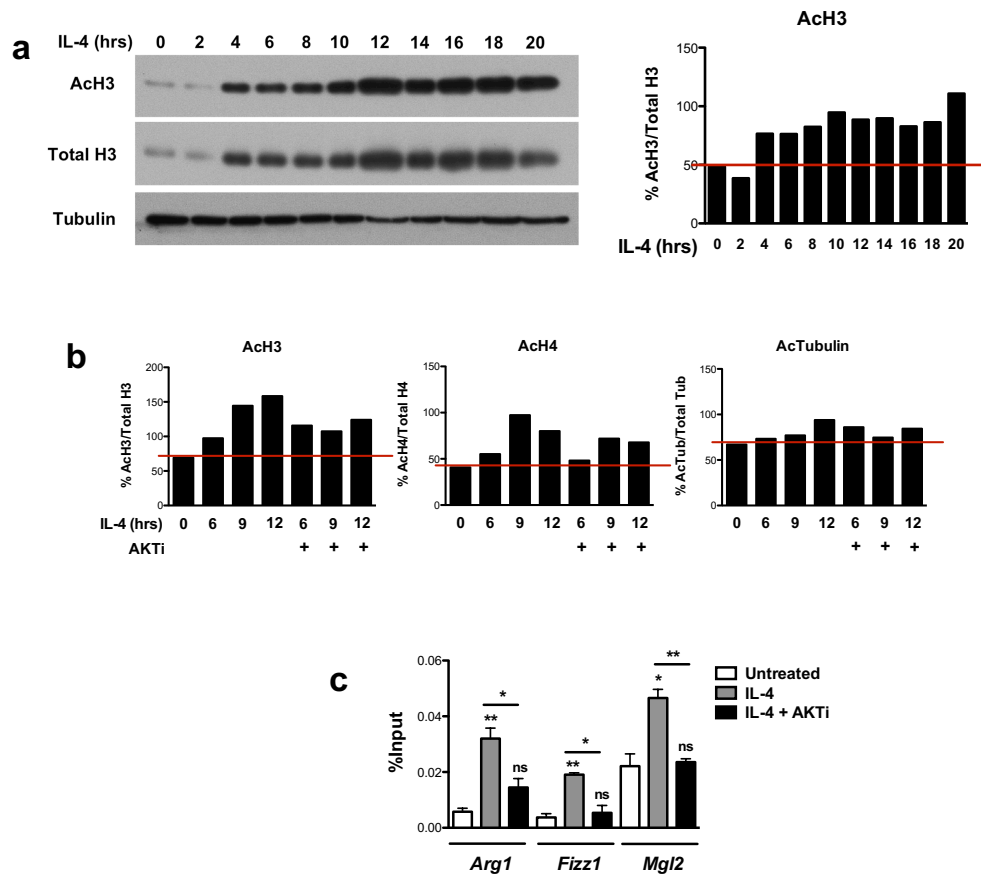
Figure 3-4. Akt regulates inducible histone acetylation at some M2 genes

A) IL-4 induces a global increase in histone H3 acetylation. BMDMs were stimulated with IL-4 over the time course indicated, followed by analysis of global histone H3 acetylation by western blotting. Right, quantitation of acetylated H3 over total H3. (AC)

B) Quantitation of the western blotting data in Figure 3-3C. (AC)

C) Akt regulates Pol II recruitment at some M2 genes. BMDMs stimulated as indicated were subject to ChIP analysis using antibodies to Pol II. Enrichment of the indicated M2 gene promoters was assessed by qRT-PCR. (AC)

The student's t-test was used to determine statistical significance, defined as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.
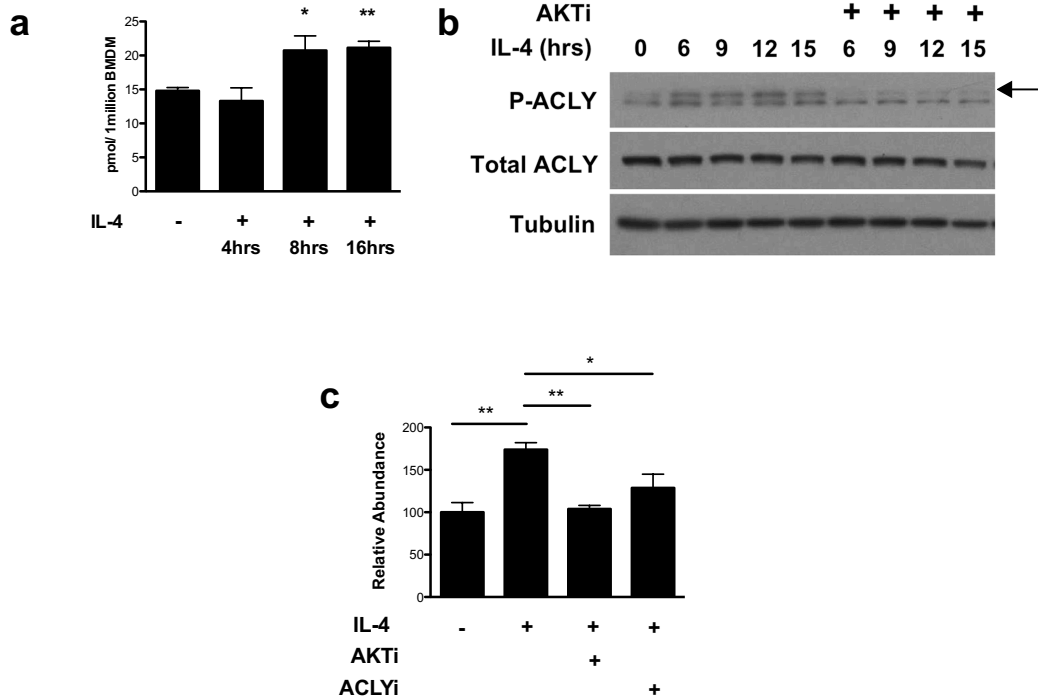
Figure 3-5. The Akt-Acly axis controls inducible Ac-CoA production in M2 macrophages

A) L-4 treatment increases Ac-CoA production. BMDMs were stimulated for the indicated time periods with IL-4, followed by analysis of Ac-CoA levels by LC-MS. (AC, NS, AW)

B) Akt regulates IL-4-inducible Acly phosphorylation. BMDMs were stimulated as indicated, followed by analysis of Acly phosphorylation by western blotting. Arrow indicates phospho-Acly. (AC)

C) Akt and Acly regulate IL-4-inducible production of Ac-CoA. BMDMs stimulated as indicated were analyzed for levels of Ac-CoA by LC-MS. (AC, NS, AW)

The student's t-test was used to determine statistical significance, defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$.
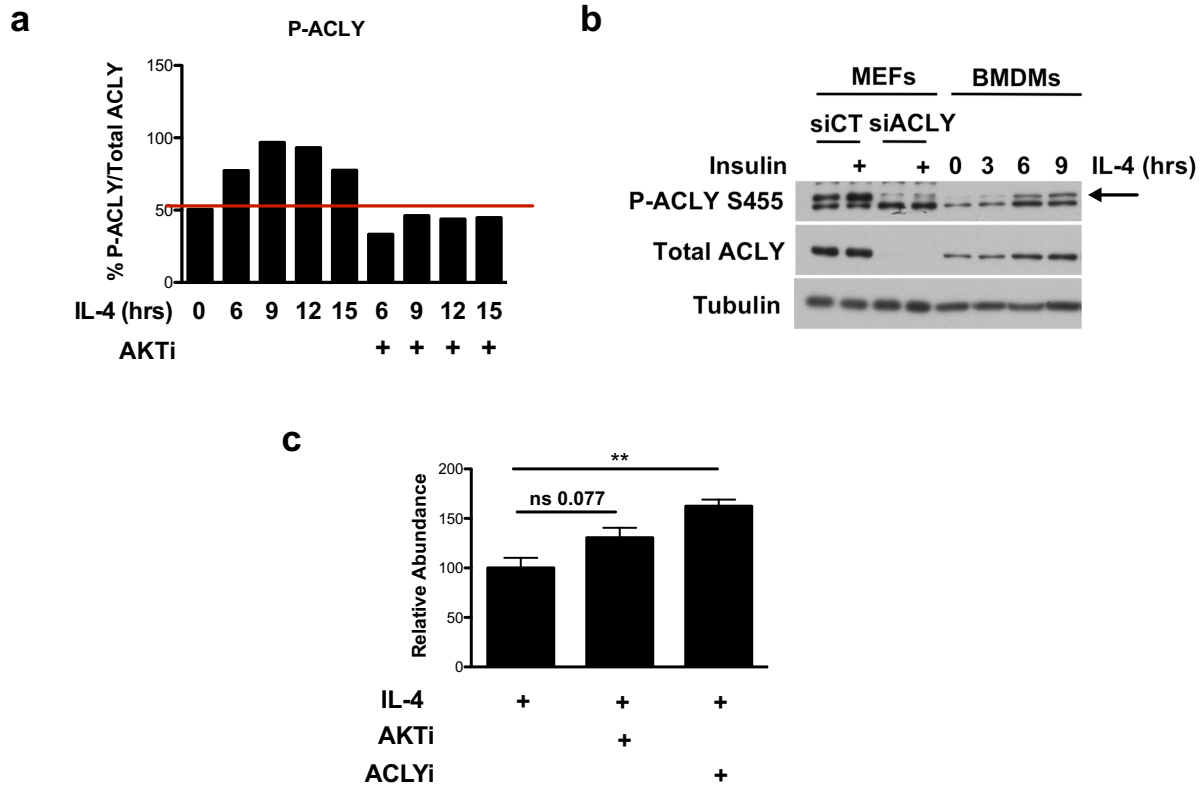
Figure 3-6. Akt-mTORC1 signaling regulates Acly to control inducible Ac-CoA production in M2 macrophages

A) Quantitation of the Acly phosphorylation from Fig 3B. (AC)

B) Knockdown of Acly in MEFs. Lysates from MEFs transfected with control siRNA (siCT) or siRNA to Acly (siAcly) were run next to BMDM lysates to unequivocally identify the bands corresponding to p-Acly and total Acly. (IBS, AC)

C) Citrate accumulates upon block of Akt or Acly activity. Citrate levels in BMDMs treated as indicated were determined by steady state metabolomics as described in Fig 1A.

The student's t-test was used to determine statistical significance, defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$. (AC, NS, AW)
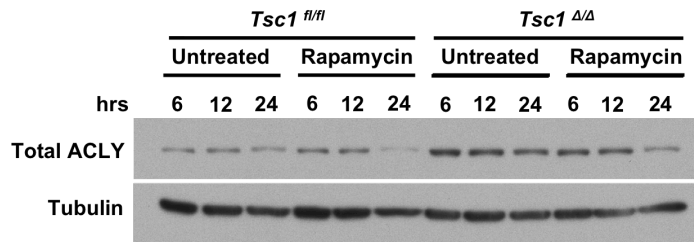
**Figure 3-7**. **mTORC1 activity regulates protein levels of Acly.** Tsc1$^{fl/fl}$ and Tsc1$^{\Delta/\Delta}$ BMDMs were treated with rapamycin or not for the indicated time periods, followed by analysis of levels of Acly by western blotting. (AC)

to a time dependent increase in Ac-CoA levels that peaked at ~8-16h, reaching a stable

increase of ~40-75% (Figure 3-5A, C). A key regulator of Ac-CoA production is the enzyme

Acly, which cleaves cytosolic citrate to produce a nuclear-cytoplasmic pool of Ac-CoA(19). Akt

has been shown to phosphorylate and activate Acly(20, 21), and we found that in M2

macrophages, IL-4 treatment stimulated the activating phosphorylation of Acly in an Akt-

dependent manner (Figure 3-5B, Figure 3-6A**).** Use of lysates from MEFs transfected with Acly

siRNA confirmed specificity in detection of phosphorylated and total Acly (Figure 3-6B).

Importantly, cotreatment with Akt or Acly inhibitors blocked the IL-4-mediated increases in Ac-

CoA levels (Figure 3-5C), indicating Akt- and Acly-mediated control of Ac-CoA production in M2

macrophages. Conversely, citrate, the substrate for the Acly reaction, accumulated in the

presence of the inhibitors (Figure 3-6C).

Our findings that Akt regulates Acly activity to control Ac-CoA production led us to

consider a role for mTORC1 in this process. mTORC1 is a key downstream effector of Akt

signaling and their activities are intricately linked in many settings, including in M2 macrophages

((11, 22-24) and Figure 3-2B). Indeed, mTORC1 is known to stimulate Acly expression(25, 26).

Previously we have used *TSC1Δ/Δ* BMDMs to investigate a role for mTORC1 in macrophage

activation; as in other cell types lacking this negative regulator of mTORC1, *TSC1Δ/Δ* BMDMs

have constitutive mTORC1 activity(11). Here we showed that Acly levels were constitutively

elevated in *TSC1Δ/Δ* BMDMs. Such elevation was sensitive to the mTOR inhibitor rapamycin,

implicating mTOR activity in regulating levels of Acly protein (Figure 3-7). Taken together, our

results indicate that the Akt-mTORC1 axis controls the activating phosphorylation and levels of

Acly to regulate Ac-CoA production in M2 macrophages.

Acly regulates gene-specific histone acetylation to control M2 activation

These data prompted us to investigate a role for Acly in M2 activation. Indeed, the Acly

inhibitor SB-204990 reduced IL-4-mediated induction of Akt-dependent M2 genes (Arg1, Fizz1,

Mgl2) but not Akt-independent M2 genes (Ym1, Fabp4, Cd36) (Figure 3-8A). The structurally

distinct Acly inhibitor MEDICA 16 had similar effects, indicating specificity in inhibition (data not

shown). Moreover, SB-204990 treatment attenuated IL-4-mediated increases in H3 and H4

acetylation at promoters of Akt-dependent M2 genes, but not Akt-independent M2 genes (Figure

3-8B, Figure 3-9A). Likewise, SB-204990 treatment diminished Pol II recruitment at Akt-

dependent M2 genes (Figure 3-9B).

Because Akt and Acly regulate a global increase in Ac-CoA levels (Figure 3-5C) but

control inducible histone acetylation only at some M2 gene promoters (Figure 3-3D, 3-3E, 3-8B,

and 3-9A**)**, Ac-CoA production is necessary but not sufficient for stimulating gene-specific

increases in histone acetylation, which must be conferred by specific transcription factors and

histone acetyltransferases (HATs). The activity of some HATs, including p300, is regulated by

Ac-CoA levels and metabolic status(27, 28). Interestingly, the p300 inhibitor anacardic acid

reduced induction of Akt-dependent but not Akt-independent M2 genes (Figure 3-8C). Similar

effects were observed with the chemically distinct p300 inhibitor C646 (data not shown).

Therefore, p300 may link the Akt/Acly-dependent rise in Ac-CoA levels to increased histone

acetylation and gene induction at some Akt-dependent M2 genes, while distinct HATs at Akt-

independent genes are insensitive to such modulation of Ac-CoA levels.

Interestingly, while Akt phosphorylation occurred within minutes of IL-4 stimulation

(Figure 3-3B), Acly phosphorylation (Figure 3-5B) and enhanced Ac-CoA levels (Figure 3-5A)

were observed with delayed kinetics (>4h). Consistently, Akt and Acly inhibitors attenuated

induction of Akt-dependent M2 genes with delayed kinetics (>4h) (data not shown). We

speculate that the lag reflects additional regulation of Acly-dependent Ac-CoA production,

including substrate availability for the synthesis of Ac-CoA.

Arginase activity is a hallmark feature of M2 activation that supports collagen production

and polyamine synthesis(17). Consistent with effects on induction of *Arg1*, arginase activity was

Figure 3-8. Acly controls inducible histone acetylation at some M2 genes
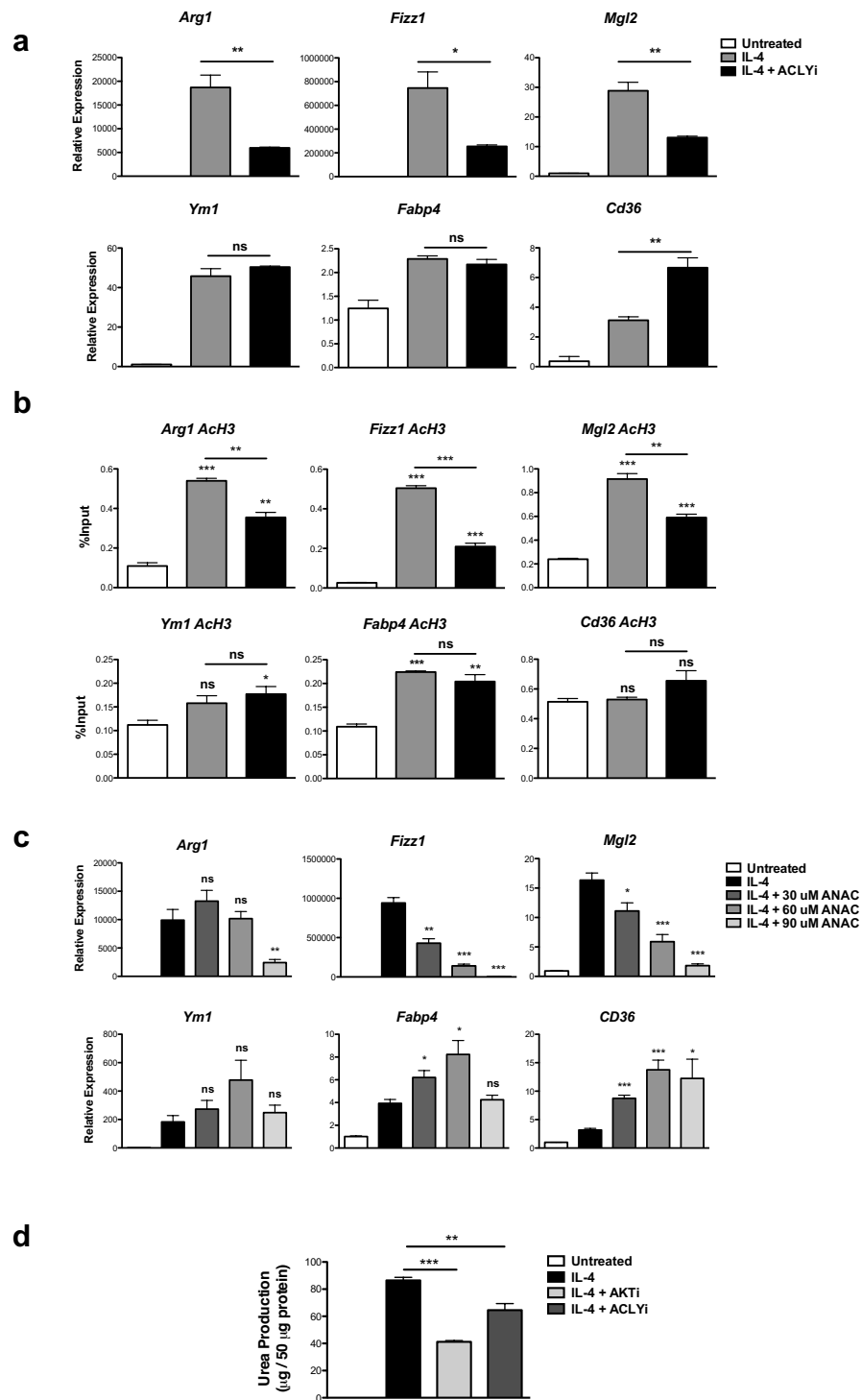
Figure 3-8 (continued)

A) Acly regulates induction of some M2 genes. BMDMs stimulated as indicated were analyzed for M2 gene induction by qRT-PCR. (AC)

B) Acly regulates inducible H3 acetylation at some M2 genes. BMDMs stimulated as indicated were subject to ChIP analysis using antibodies to acetylated H3. Enrichment of the indicated M2 gene promoters was assessed by qRT-PCR. (AC)

C) The p300 inhibitor anacardic acid (ANAC) reduces induction of some M2 genes. BMDMs stimulated as indicated were analyzed for M2 gene induction by qRT-PCR. (TS)

D) Akt and Acly control IL-4-inducible arginase activity. BMDMs were stimulated as indicated, followed by analysis of arginase activity in cellular lysates as assessed by urea production. (AC) The student's t-test was used to determine statistical significance, defined as *$P<0.05$, **$P<0.01$, and ***$P<0.001$.
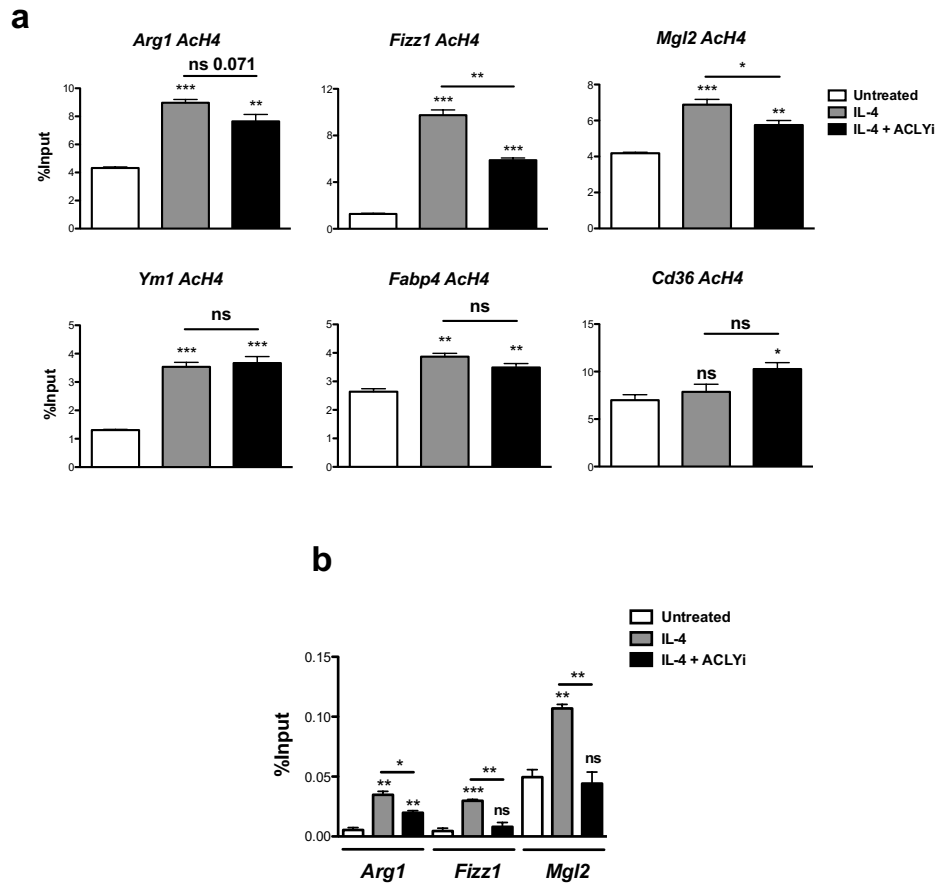
Figure 3-9. Acly controls inducible histone acetylation at some M2 genes

A) Acly regulates inducible H4 acetylation at some M2 genes. BMDMs stimulated as indicated were subject to ChIP analysis using antibodies to acetylated H4. Enrichment of the indicated M2 gene promoters was assessed by qRT-PCR. (AC)

B) Acly regulates Pol II recruitment at some M2 genes. BMDMs stimulated as indicated were subject to ChIP analysis using antibodies to Pol II. Enrichment of the indicated M2 gene promoters was assessed by qRT-PCR. (AC)

The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.
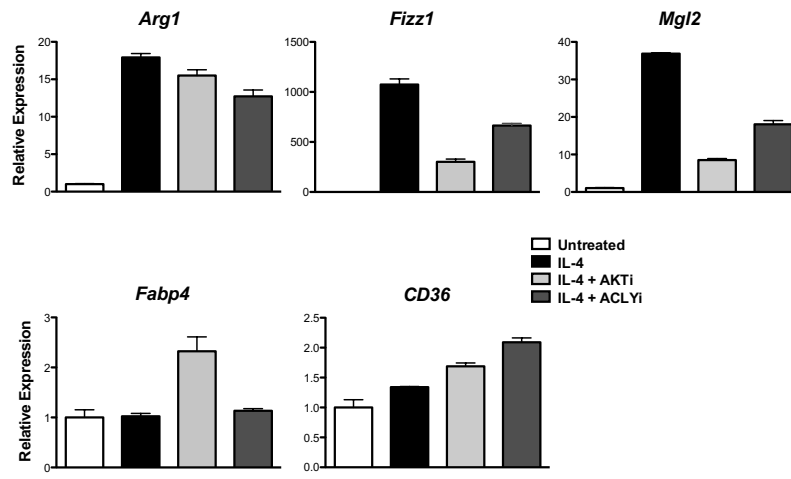
Figure 3-10. Akt-Acly signaling regulates M2 activation in peritoneal macrophages.

Elicited peritoneal macrophages were treated with IL-4 +/- the indicated inhibitors, followed by

analysis of M2 gene induction by qRT-PCR. (JY, AC)

regulated by Acly and Akt (Figure 3-8D). Additionally, Akt and Acly inhibitors reduced induction

of Akt-dependent M2 genes in peritoneal-elicited macrophages, indicating that control of M2

activation by the Akt-Acly axis may be applicable to multiple macrophage populations (Figure 3-

10).

The Akt-mTORC1 pathway couples metabolic input to induction of some M2 genes

The Akt-mTORC1 pathway is a major metabolic sensor, and mTORC1 activity in

particular is controlled by amino acid levels, ADP/ATP levels, and other metabolic inputs(22, 23).

Therefore, we considered that incorporation of the Akt-mTORC1 pathway into IL-4 signaling,

parallel to canonical Jak-Stat signaling, may allow particular subsets of the M2 transcriptional

program to integrate signals reflecting the cellular metabolic state (Figure 3-11A). Amino acids

directly and potently regulate mTORC1 activity independent of the TSC complex(22, 23) and

can also activate Akt in some contexts(29, 30), hence we varied amino acid concentrations as a

way to modulate Akt-mTORC1 activity. As expected, mTORC1 activity, as assessed by

phosphorylation of its downstream target S6K, was greatly reduced in amino acid deficient

media and intermediate in media containing low levels of amino acids (Figure 3-11B). In line

with(29, 30), increasing amino acid levels also augmented Akt activation, as indicated by

enhanced phosphorylation on two critical residues, T308 and S473 (Figure 3-11B). Titrating

amino acids had no effect on Stat6 phosphorylation and activation (Figure 3-11B), validating the

use of this experimental model to modulate the Akt-mTORC1 axis independent of canonical

Stat6 signaling. Consistent with effects on mTORC1 and Akt activity, amino acid levels dose

dependently increased Acly phosphorylation and protein levels (Figure 3-11B) as well as Ac-

CoA production (Figure 3-11C). Importantly, amino acids potentiate induction of Akt-dependent

M2 genes but not Akt-independent M2 genes (Figure 3-11D).

We also examined M2 activation using the complementary model of leucine deprivation,

since leucine is particularly critical in regulation of mTORC1 activity(31). Here comparisons
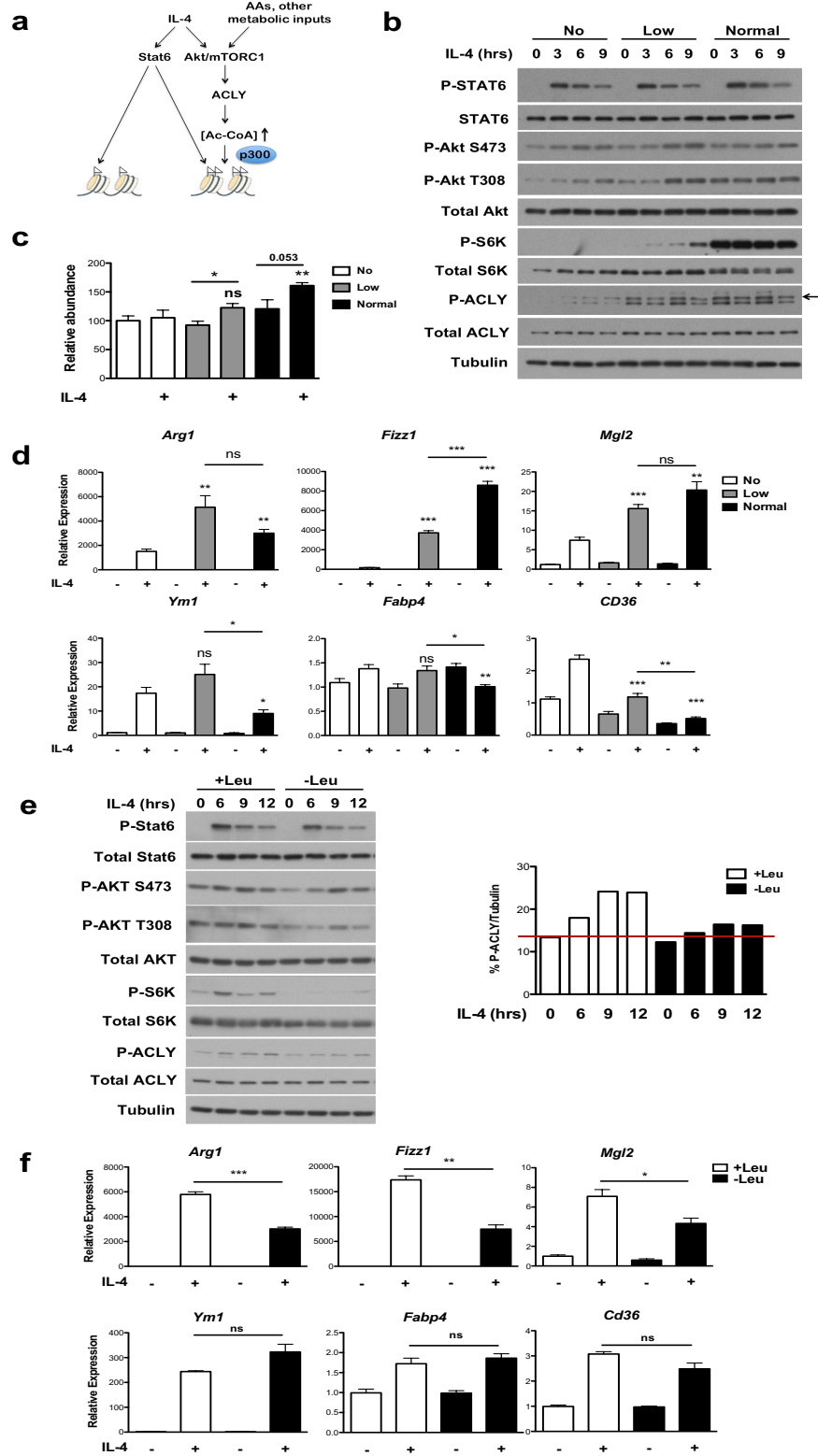
Figure 3-11. The Akt-mTORC1-Acly axis links metabolic input to control of M2 activation

Figure 3-11 (continued)

A) Proposed model for how Akt-mTORC1-Acly signaling exerts gene-specific control of M2 activation. Akt-TORC1-Acly signaling integrates metabolic input to control levels of Ac-CoA production, which modulates histone acetylation and gene induction at some M2 genes by HATs such as p300. (TH)

B) Amino acid levels modulate the activity of the Akt-mTORC1-Acly axis. BMDMs cultured in media containing varying levels of amino acids (normal, low, or no) were stimulated with IL-4 for the indicated time periods, followed by analysis of Akt, mTORC1, and Acly activity by western blotting. (AC)

C) Amino acid levels modulate Ac-CoA production. BMDMs stimulated as in B. were analyzed for Ac-CoA levels by LC-MS. (AC, NS, AW)

D) Amino acid levels modulate induction of some M2 genes. BMDMs stimulated as in B. were analyzed for M2 gene induction by qRT-PCR. (AC)

E) Leucine deficiency attenuates the activity of the Akt-mTORC1-Acly axis. BMDMs cultured in leucine-replete or leucine-deficient media were stimulated with IL-4 for the indicated time periods, followed by analysis of Akt, mTORC1, and Acly activity by western blotting. *Right,* quantitation of Acly phosphorylation. (AC)

F) Leucine deficiency reduces induction of some M2 genes. BMDMs stimulated as in E. were analyzed for M2 gene induction by qRT-PCR. (AC)

The student's t-test was used to determine statistical significance, defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$.
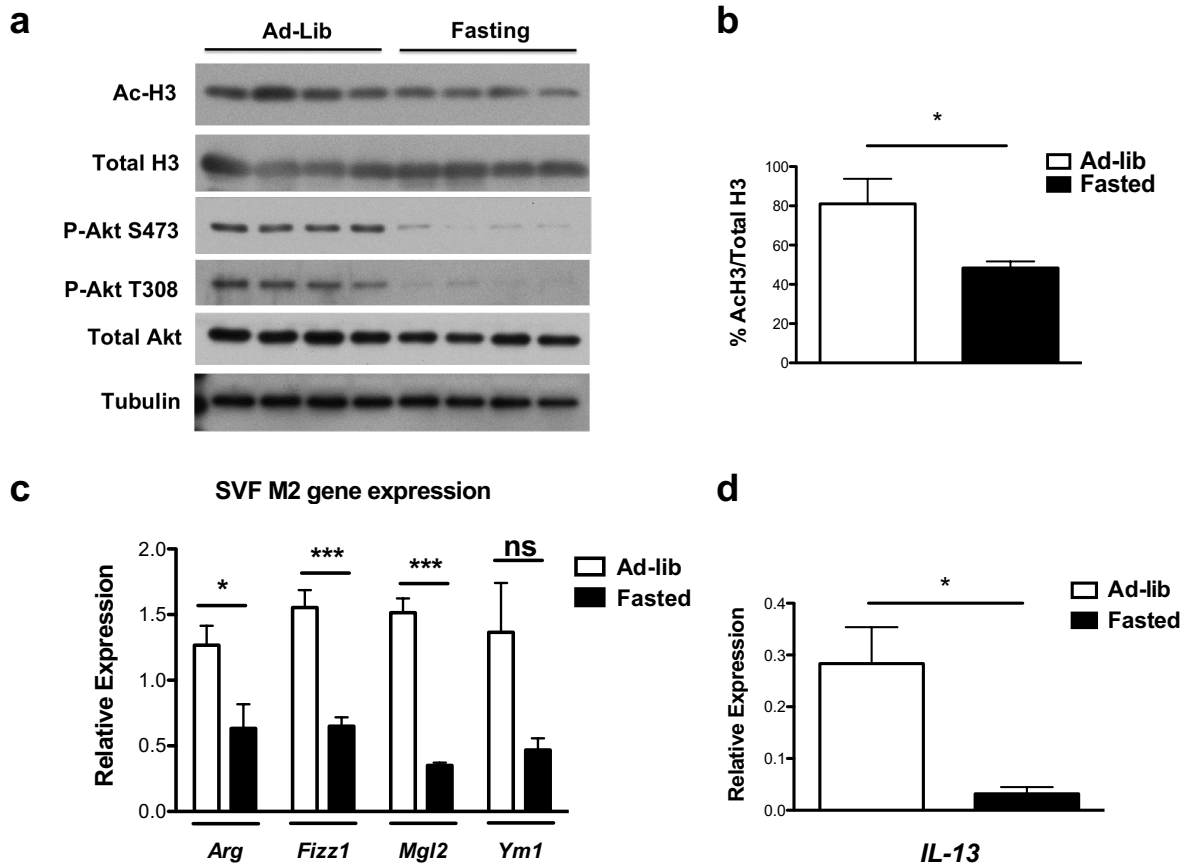
Figure 3-12. Feeding and fasting regulate M2 polarization of adipose tissue macrophages.

Analysis of ATM M2 activation in fed and fasted mice. Mice (n=4/group) were allowed to feed ad-lib or fasted O/N prior to their sacrifice.

A-B) The ATM-containing stromal vascular fraction of perigonadal white adipose tissue was obtained for analysis of Akt activity and H3 acetylation by western blotting. (AC)

C) Expression of M2 genes in the SVF of fed and fasted mice by qRT-PCR. (AC)

D) Expression of IL-13 in whole adipose tissue of fed and fasted mice by qRT-PCR. (AC)

The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.

were made between culture conditions that differed only in the presence or absence of one amino acid, without significant effects on total levels of amino acids. Culture in leucine-deficient media attenuated IL-4-inducible mTORC1 and Akt activity and Acly phosphorylation, but not Stat6 phosphorylation (Figure 3-11E). Importantly, leucine deficiency selectively reduced expression of Akt-dependent M2 genes (Figure 3-11F). Taken together, these results indicate that amino acids and likely other metabolic inputs feed into the Akt-mTORC1 axis to calibrate M2 activation to the metabolic state (Figure 3-11A).

Finally, we found that physiological changes to nutrient levels can modulate M2 activation in adipose tissue macrophages (ATMs). ATM M2 polarization is thought to critically maintain insulin sensitivity in white adipose tissue, so such feeding-induced increases in M2 activation may coordinate responses to nutrient influx to mediate metabolic homeostasis in the postprandial state(18). Specifically, we found that Akt activation was increased in the fed state compared to the fasted state in the ATM-containing stromal vascular (SVF) fraction of the white adipose tissue (Figure 3-12A). Interestingly, global H3 acetylation and M2 gene expression followed a similar pattern and were elevated in the fed state (Figure 3-12A, B, C). Expression of all M2 genes was elevated in the fed state (Figure 3-12C), consistent with an important role for IL-13, a critical regulator of ATM M2 polarization(18) that is increased in the fed state (Figure 3-12D), in feeding-induced ATM polarization, although postprandial elevations in nutrients like amino acids and glucose may also contribute. Therefore, the Akt-mTORC1-Acly pathway may regulate enhanced M2 activation of ATMs in the fed state.

Akt and Acly regulate functional subsets of the M2 program

We employed genome wide transcriptional profiling to obtain a comprehensive view of regulation of M2 activation by the Akt-Acly pathway. BMDMs were treated for 16 h with IL-4 with or without Akt or Acly inhibitors, followed by RNA seq (Figure 3-13) or microarray analysis (data not shown). In the RNA seq analysis, 758 genes were induced >2.0 fold by IL-4, of which

91 were downregulated >30% by both Akt and Acly inhibitors (including Arg1, Fizz1, and Mgl2), confirming critical roles for Akt and Acly in control of M2 activation as well as substantial overlap in the activities of the two proteins (Figure 3-13A and 3-13B). A subset of Akt inhibitor sensitive genes was sensitive to Acly inhibitor (91/327), in line with a broader role for Akt in control of cell physiology. In contrast, most genes sensitive to Acly inhibitor were sensitive to Akt inhibitor (91/118). This indicates that in the context of M2 activation, Acly is a major target of Akt and is critically controlled by Akt activity, likely in regulation of Ac-CoA production and histone acetylation at M2 genes (Figure 3-13A, B).

Gene enrichment analysis of the 91 Akt- and Acly-coregulated genes identified preferential enrichment of several pathways, including cell cycle and DNA replication (Figure 3-13C, D). IL-4 triggered BrdU labeling of a subset of BMDMs in vitro (data not shown) and proliferation of macrophages in vivo(15), thus IL-4 may stimulate macrophage proliferation in an Akt- and Acly-dependent manner. Consistently, metabolic processes underlying cellular proliferation were among the top enriched pathways in our metabolomics analysis, including nucleotide metabolism and protein biosynthesis (Figure 3-1A). Interestingly, chemokines were also enriched in Akt- and Acly-coregulated genes (Figure 3-13C, D, E), including Ccl2, Ccl7, Ccl17, and Ccl24. Finally, genes in the eosinophil associated ribonucleases (Ear) family were found to be regulated by Akt and Acly. While they barely miss the stringent cutoffs that we set, qPCR analysis confirmed coregulation of Ear2, Ear11, and Ear12 genes by the Akt-Acly pathway (Figure 3-13E). Ear genes are of interest because Ear2 and Ear11 are thought to have chemoattractant activity for dendritic cells and macrophages and are known to be highly induced in settings of Type 2 inflammation(32, 33). We also performed gene enrichment analysis on the 404 IL-4 inducible genes that were not regulated by either Akt or Acly, and identified keratin sulfate biosynthesis, cysteine and methionine metabolism, and other pathways (Figure 3-13C, D). Thus, the transcriptional profiling analysis indicates that the Akt-Acly
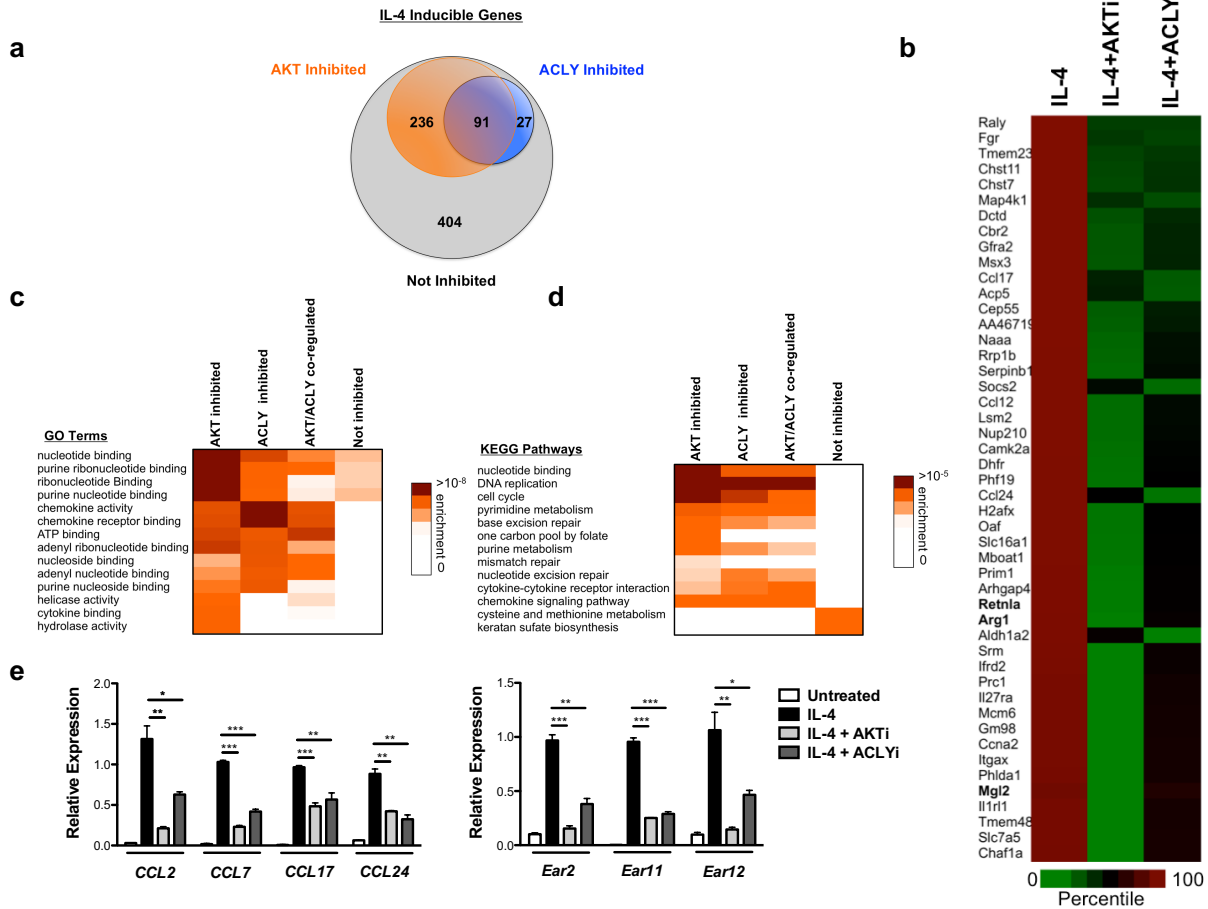
Figure 3-13. The Akt-Acly axis controls functional subsets of the M2 program

A) Venn diagram depicting the number of IL-4-inducible genes regulated by Akt and/or Acly signaling. (SI, AC)

B) Heatmap of normalized rank ordered Log2 RPKM values of top 50 IL-4 response genes co-regulated by Akt and Acly. (SI, AC)

C) Heatmap of enriched KEGG pathways within the cohort of IL-4-inducible genes. (SI, AC)

D) Heatmap of enriched Gene Ontology terms within the cohort of IL-4-inducible genes. (SI, AC)

E) qPCR analysis validates regulation of chemokine and Ear genes by Akt-Acly signaling. (AC) The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.
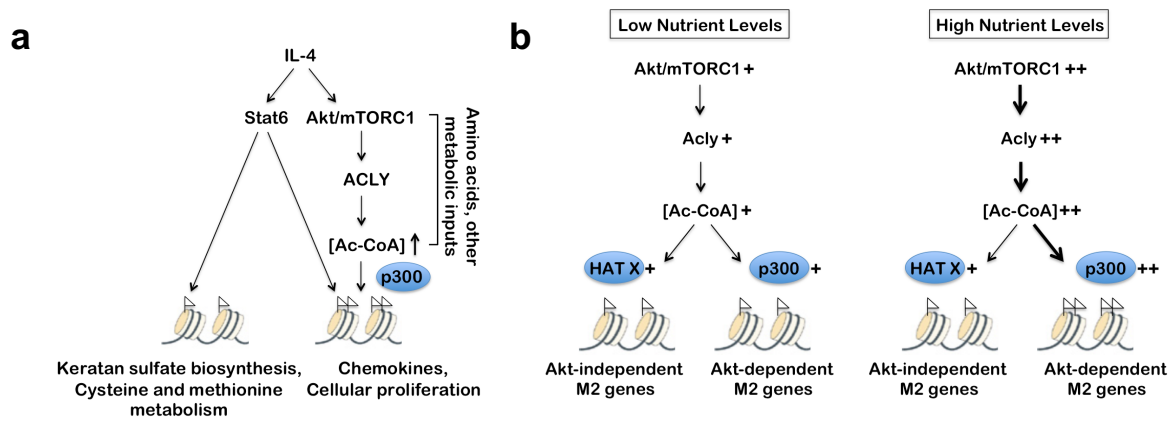
Figure 3-14. Working models

A) The Akt-mTORC1-Acly pathway couples metabolic input to control of particular subsets of the M2 program, including chemokines and cellular proliferation. (TH)

B) p300 links metabolic status and Akt-mTORC1 activity in the form of Ac-CoA levels to M2 gene induction. p300 has a high Km and is relatively sensitive to levels of its substrate, allowing it to couple increased Ac-CoA levels to enhanced histone acetylation at some Akt-dependent M2 genes. In contrast, distinct HATs at Akt-independent M2 genes have low Km and are relatively insensitive to metabolic status and changing Ac-CoA levels. (TH)

pathway controls selective subsets of the M2 program to allow their modulation by metabolic input (Figure 3-14A, B). As M2 macrophages play a key role in metabolic homeostasis, parasite infection, allergic diseases, and wound healing and tissue repair(17, 18), these findings are relevant for metabolic control of macrophage function in diverse contexts.

Discussion

The Akt-mTORC1 pathway has a well-established role in promoting anabolic metabolism in growing/proliferating cells, tumor cells, and metabolic tissues. In the context of cellular proliferation, for example, Akt-mTORC1 activity couples growth factor signaling and nutrient availability to the synthesis of proteins, lipids, and nucleotides(23). In stark contrast, the role of the Akt-mTORC1 pathway in macrophages is much less intuitive. What is the teleological rationale for control of macrophage activation by Akt-mTORC1 signaling (and metabolism more generally)? Here we show that IL-4 signaling leads to parallel and independent activation of the Akt-mTORC1 pathway and the canonical Jak-Stat pathway. This allows the Akt-mTORC1 axis to regulate a subset of M2 genes through control of Acly activity/expression, Ac-CoA production, and histone acetylation. We propose that IL-4 signaling *co-opted* the Akt-mTORC1 pathway to couple metabolic input to epigenetic regulation of certain components of the M2 response, including chemokines and cellular proliferation (Figure 3-14). As discussed above, Akt-mTORC1 signaling acts as a metabolic checkpoint in the context of cellular division, allowing growth and proliferation only when nutrients are abundant. Chemokines are key in amplifying inflammation, and we propose that their modulation by this pathway enables metabolic input to calibrate the strength and duration of energetically costly immune responses(34). These findings add a new dimension to our emerging understanding of how macrophage metabolism supports macrophage activation.

As discussed above, the Akt/Acly-dependent rise in Ac-CoA production is necessary but not sufficient for stimulating gene-specific increases in histone acetylation. Our data suggests

that at some Akt-dependent M2 genes, p300 may link metabolic status and Akt/mTORC1 activity, in the form of Ac-CoA levels, to histone acetylation and transcriptional induction (Figure 3-14). p300 has a high K_m, which may allow it to couple increased Ac-CoA levels to enhanced histone acetylation(27, 28). In contrast, distinct HATs at Akt-independent M2 genes may have a low K_m and are thus insensitive to such modulation of Ac-CoA levels. Presumably, differential HAT recruitment is mediated by distinct transcription factors at Akt-dependent and independent M2 genes, which would be important to address in future studies.

Although Akt activity has been linked to M2 activation, the role of mTORC1 remained less clear, due in part to some caveats and intractabilities of genetic and pharmacological tools to block mTORC1 activity(35). Here, use of amino acids to modulate Akt-mTORC1 signaling suggests that mTORC1 activity supports M2 gene induction (Figure 3-11). Previously, we and others have shown that in BMDMs lacking TSC1, a negative regulator of mTORC1, aberrantly increased mTORC1 activity attenuates induction of M2 genes(11, 36). We hypothesize that the difference between the two models reflects divergent control of M2 activation by physiological and pathophysiological mTORC1 activity respectively. While this idea remains to be tested, the context-dependent roles of mTORC1 in insulin signaling is well-established. In lean/healthy animals, mTORC1 critically mediates insulin signaling in metabolic tissues (to coordinate postprandial nutrient storage), but in obesity, chronic nutrient excess leads to an aberrant increase in mTORC1 activity that contributes directly to insulin resistance and metabolic dysregulation(22).

Metabolic status has long been proposed to modulate epigenetic control of gene expression(37-39), but only recently have a handful of studies linked physiological changes in metabolite levels to epigenetic regulation of gene expression(19, 21, 40-44). Here we show how the Akt-mTORC1 axis couples metabolic input in the form of Ac-CoA levels to histone acetylation and gene regulation, and importantly, to control specific subsets of the M2 program.

In addition to a recent study(21), this is only the second example of how Akt-Acly signaling controls gene regulation through histone acetylation. Other macrophage polarizing signals and common gamma chain cytokines (γ_c) (e.g. IL-2, IL-15) engage the Akt-mTORC1 axis, thus our findings may have implications for multiple programs of macrophage polarization and leukocyte activation. Canonical signaling downstream of the polarizing signal or g_c specifies which genes are induced, while regulation of Ac-CoA levels and histone acetylation by the Akt-mTORC1-Acly pathway allows metabolic input to calibrate genes encoding energetically demanding processes. Alternatively, Ac-CoA can be synthesized independently of the Akt-Acly axis by AceCS1(45) or nuclear pyruvate dehydrogenase(46) to mediate histone acetylation. AceCS1 activity is controlled by SIRT1, thus linking this pathway for Ac-CoA production and histone acetylation to conditions of low energy or nutrients(45). Therefore, future studies to determine how gene-specific histone acetylation is regulated during different macrophage activation programs are warranted. These studies could pave the way towards new therapeutic approaches of modulating macrophage function in diverse contexts, including Type 2 inflammation, metabolic homeostasis, and antimicrobial immunity.

Materials and Methods

BMDM culture and stimulations.

BMDM cultures were established as described(11). For stimulations, BMDMs were pretreated for 1 h with inhibitors followed by addition of 10 ng/ml IL-4 for 16 h unless otherwise indicated. Inhibitors were used as follows: AKT inhibitor MK-2206, 2-5 μM (Selleck); ACLY inhibitor SB-204990, 40 μM (Tocris); etomoxir, 200 μM (Sigma), and 2-deoxy-glucose, 1 mM (Sigma). For amino acid titration experiments, BMDMs were plated in DMEM containing low levels of amino acids for 6 hours (to deplete cellular amino acid pools) prior to changing the media to DMEM with varying levels of amino acids (no, low, or normal) +/- IL-4 for 16 hours. Normal is normal tissue culture media, while low indicates media containing 5% of the normal levels of amino

acids (obtained by mixing normal media and media lacking amino acids). In experiments with leucine free media, BMDMs were stimulated in complete DMEM or –Leu complete DMEM (Crystalgen) +/- IL-4 for 16 hours. *TSC1*D/D BMDMs were described previously(11).

Mice.

C57BL/6 mice were used for in vivo studies and as a source of BMDMs. Mice were maintained at Harvard Medical School and all procedures were performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committees at the institution.

Immunoblotting.

Cells were lysed directly in 6X SDS loading buffer (histone western blots) or in 1% NP-40 buffer (all other western blots). Protein concentration was determined using the Bradford method. Primary antibodies were purchased from Cell Signaling except for a-Tubulin (Sigma), acetylated Tubulin (Sigma), acetylated H3 (Millipore), acetylated H4 (Millipore), and total H4 (Abcam).

Arginase assay.

Arginase assay was done as described(11).

Extracellular flux assays.

Oxygen consumption and extracellular acidification rates were measured with a XF96 extracellular flux analyzer (Seahorse Bioscience). Seahorse assay media containing 11 mM glucose or plain assay media was used for the mitochondrial and glycolysis stress tests respectively. OCR measurements were taken before and after the sequential addition of 1 μM oligomycin, 1.5 μM FCCP and 2 μM antimycin/rotenone. ECAR measurements were taken

before and after the sequential addition of 11 mM glucose, 1 μM oligomycin and 0.5 M 2-DG

(Sigma). Values were normalized with Hoechst 33342 staining (Life Technologies).

Glucose uptake.

BMDMs were washed with Krebs-Ringer bicarbonate HEPES (KRBH) buffer once, followed by

addition of 400 μl KRBH buffer. 100 μl loading buffer (KRBH buffer with 0.5 mM 2-deoxy-D-

glucose (Sigma) and 1 μCi/well ^{3}H-deoxy-D-glucose (2-^{3}H(G)) (PerkinElmer, 1 mCi/ml in

EtOH:water (9:1)) was added and incubated at 37C for exactly 15 min. 20 μl stop solution (1.5

mM cytochalasin B (Sigma) in DMSO) was added, and the cells were washed with KRBH buffer

before lysis in 0.1 N NaOH. The glucose uptake rate was determined by normalizing cellular

^{3}H-deoxy-D-glucose count to protein concentrations.

Fatty acid oxidation.

Fatty acid oxidation was done as described(11).

Chromatin immunoprecipitation.

ChIP was done as described(11), using acetylated H3 (Millipore 06-599), acetylated H4

(Millipore 06-866), or IgG (Santa Cruz SC-2027) antibodies. Fold enrichment was calculated as

ChIP signals normalized to input.

Gene expression.

RNA was isolated using RNA-Bee (Tel-Test) per manufacturers protocol. cDNA synthesis was

done using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A Bio-Rad

C1000 Thermocycler was used for qPCR, and data was analyzed by means of the CFX Manger

Software (Bio-Rad) using the delta/delta CT method. BMDM samples were normalized to

hypoxanthine phosphoribosyltransferase and relative fold change was calculated by setting the

basal state (untreated) to 1 and all other conditions are relative to the basal state for each gene

analyzed. Ex vivo samples were normalized to the macrophage marker CD68.

Dual luciferase assays.

BMDMs were electroporated using mouse macrophage nucleofector kit (Lonza) and the Amaxa

machine with STAT6-Firefly luciferase (Addgene plasmid #35554) along with Renilla–Luciferase

plasmid as a transfection control. BMDMs were stimulated with or without 10 ng/ml IL-4 4 h

post electroporation for another 24 h. Cell lysates were collected and analyzed using the

Promega Dual-Luciferase Reporter Assay System.

Acyl-CoA Mass Spectrometry.

BMDMs were lysed in 800 μl ice cold 10% TCA (Tricholoracetic acid). Sc5-sulfosalicylic acid

(SSA), ammonium formate, $[^{13}C_6]$-glucose, sodium $[^{13}C_{16}]$-palmitate, and analytical standards for

acyl-CoAs were from Sigma-Aldrich (St. Louis, MO). Optima LC-MS grade methanol,

ammonium acetate, acetonitrile (ACN) and water were purchased from Fisher Scientific

(Pittsburgh, PA). Calcium $[^{13}C_3^{15}N_1]$-pantothenate was purchased from Isosciences (King of

Prussia, PA). $[^{13}C_3^{15}N_1]$-acyl-CoA internal standards for quantitation were generated by pan6

deficient yeast culture as previously described(47), with 100 µL of extract spiked into samples

before extraction. Standard curves were prepared using the same batch of internal standard,

and all samples were extracted by solid phase extraction as previously described(48). Acyl-

CoAs were analyzed as previously described for quantitation(49) and for isotopolog analysis(50)

by liquid chromatography-tandem mass spectrometry on an Agilent 1200 coupled to an

API4000 in the positive ion mode monitoring the acyl-CoA specific neutral loss of 507 amu from

each acyl-CoA, internal standard and isotopolog.

Steady state metabolomics.

BMDMs were stimulated for 10 h with IL-4 before media was refreshed by addition of complete

RPMI with IL-4 for another 2 h. Preparation of cellular extracts was done as described(16).

Steady state metabolomics was done at Beth Israel Deaconess Medical Center Mass

Spectrometry Facility. Data analysis was performed as described(16).

RNA-seq Library Construction, Mapping, and Analysis.

Strand-specific libraries were generated using 500ng RNA input using TruSeq library

preparation kit (Illumina, San Diego, CA). cDNA libraries were multiplexed using specific unique

adaptors and sequenced using Illumina NextSeq 500 under single end 75bp read length

parameters. Reads were aligned to the mouse mm10 reference genome using TopHat using

default settings(51). Alignments were restricted to uniquely mapping reads, with up to 2

mismatches permitted. RPKM was calculated as described for mm10 Refseq genes by

counting exonic reads and dividing by mRNA length(52). Coexpressed gene classes were

generated with Cluster3 by applying k-means clustering to mean-centered $log2$(FPKM)

expression values. Differential analyses was performed using DEseq(53) using default

parameters for the indicated comparisons. Cohort of IL-4 inducible genes was defined by

following: >2 RPKM, Log2fold>1.0, DESeq p-adj<0.05 yielding 758 IL-4 inducible genes.

Inhibition by AKT or ACLY inhibitors defined as 30% reduction in RPKM and DESeq p-adj <0.05.

Enrichment of KEGG pathways and Gene Ontology (GO) terms analysis performed using

DAVID(54).

Feeding/fasting experiments.

8-10 week old C57BL/6 mice were fasted overnight or allowed to feed ad-libitum. Mice were sacrificed the next morning and the perigonadal adipose tissue was excised. A small section of whole adipose tissue (WAT) was homogenized in RNA-Bee for analysis of gene expression in unfractionated WAT. The remaining adipose tissue was minced and digested in 5 ml Krebs ringer buffer (KRBH) containing 2% fatty acid free BSA and 2 mg/ml collagenase (Sigma, C2674) for 20 mins at 37°C. The resulting cell suspension was filtered through a 250 mm nylon mesh and centrifuged at 1200 RPM to obtain a cell pellet corresponding to the stromal vascular fraction (SVF), which was lysed for RNA extraction or western blotting.

Statistical analysis.

Statistical analysis was carried out using Prism (GraphPad) software. The student's t-test was used to determine statistical significance, defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$

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Chapter 4

Discussions and Future Directions

Attenuated Akt signaling underlies aberrant macrophage polarization in *Tsc1^{Δ/Δ}* BMDMs

Over the last two decades, studies have shown that activated macrophages are able to polarize to pro-inflammatory or alternative states with distinct phenotypes and physiological responses during inflammation. As a result, macrophages have been categorized into subtypes that include the classical pro-inflammatory M1 state induced upon stimulation with Interferon-γ and LPS, and the alternative immunoregulatory M2 state by stimulation with the Th-2 cytokines IL-4 and IL-13. The M1 state is characterized by increased expression of proinflammatory cytokines IL-6, IL-12 and TNF-α, and microbicidal activity, while M2 macrophages have increased expression of anti-inflammatory cytokines and genes such as IL-10, TGF-β and Arginase1 to promote tissue remodeling, wound repair and parasitic killing [1].

The Akt-mTOR pathway is a central signaling node and nutrient sensing pathway that is able to integrate nutrient/energy status, cytokines, growth factors and cellular stress to coordinate downstream anabolic processes such as lipid, nucleotide and protein synthesis to support cellular proliferation and effector functions [2]. mTOR exists in two separate complexes, mTORC1 and mTORC2, that regulate distinct signaling pathways and phosphorylation of downstream targets. In addition to its role as a metabolic sensor, mTOR has also been shown to modulate immune responses in both innate and adaptive immune cells such as dendritic cells and T cells [3]. Recent studies by Jonathan Powell and colleagues highlight an important role for mTOR in T cell biology, with mTORC1 being necessary for the development of Th1 and Th17 cells, while mTORC2 is necessary for the development of Th2 cells [4, 5]. It has been well established that LPS treatment of macrophages activates the PI3K-Akt-mTORC1 pathway[6]: however, the consequences of this activation are incompletely understood, for example, according to Schmitz et al., inhibition of mTORC1 with rapamycin leads to increased production

of pro-inflammatory IL-1β and lethality during LPS induced endotoxin shock [7]. Conversely, other studies reported that rapamycin treatment of macrophages leads to decreased expression of pro-inflammatory genes and plays a protective role against endotoxin shock [8, 9]. Collectively, these studies provide evidence for the role of mTOR in regulating macrophage cytokine expression and inflammation, however due to the contrasting conclusions of these papers the role of mTOR in macrophage immunity remains unclear.

Our findings from Chapter 2 (Byles et al., 2013) using a mouse model with tissue specific deletion of TSC1 in myeloid cells ($Tsc1^{\Delta/\Delta}$) shows that macrophages with constitutive mTORC1 have a hyper-inflammatory response to LPS with increased gene expression of the inflammatory cytokines IL-6, TNF-α, and IL-12 [10]. Additionally, TSC1-null macrophages are defective in IL-4 dependent M2 polarization as demonstrated by decreased expression of canonical M2 markers such as Arg1, Mgl2, and Fizz1. To test M2 polarization *in vivo*, we intraperitoneally injected $Tsc1^{fl/fl}$ control mice and $Tsc1^{\Delta/\Delta}$ mice with chitin and directly with an IL-4 antibody complex and measured M2 gene expression in peritoneal exudate cells (PECs). In both models we observed a decrease in the expression of multiple M2 genes (*Arg1*, *Fizz1*, *Mgl1, Mgl2, Pgc1β*) as well as a decrease in the expression of *IL-10* in $Tsc1^{\Delta/\Delta}$ PECs. The striking reduction in the majority of the M2 genes is consistent with a role of constitutive mTORC1 activity in attenuating M2 polarization *in vivo*.

Additional experiments from Chapter 2 showed that constitutive mTORC1 leads to negative feedback mechanisms that give rise to the serine phosphorylation and degradation of IRS2, an upstream regulator of Akt activation. Consequently, TSC1-null macrophages treated with LPS or IL-4 had attenuated Akt phosphorylation/activation, consistent with the obligatory role of phosphorylation of serine 473 and threonine 308 for full Akt activation [11]. These findings suggest that attenuated Akt signaling critically underlies the defect in M2 activation and pro-inflammatory phenotype in $Tsc1^{\Delta/\Delta}$ BMDMs, and appears to be the result of mTORC1-

mediated feedback inhibition of Akt signaling. In support of this, 1) $Tsc1^{\Delta/\Delta}$ BMDMs display decreased P-Akt^{T308} and P-Akt^{S473} in response to IL-4 stimulation, 2) Akt inhibitors block M2 activation in WT cells and rapamycin treatment increases expression of M2 genes in $Tsc1^{\Delta/\Delta}$ BMDMs, and 3) ectopic expression of myr-Akt in $Tsc1^{\Delta/\Delta}$ BMDMs is sufficient to rescue expression of *Arg1* and *Mgl1* and decrease LPS induced inflammatory gene expression while increasing expression of IL-10. Furthermore, a critical role for Akt in regulating macrophage polarization is also supported by studies of *Akt1*KO macrophages, which have a pro-inflammatory phenotype and reduced levels of C/EBPβ (due to enhanced miR-155), and *Akt2*KO macrophages which leads to increased expression of M2 genes [12]. Based on these results, activation of Akt signaling by IL-4 may serve as a way to inhibit inflammatory cytokine production while simultaneously promoting M2 gene expression. Thus we revealed an unexpected role for Akt in the regulation of macrophage polarization and is the rationale for the studies in Chapter 3 aimed at deciphering how downstream targets of Akt regulate macrophage polarization.

In our proposed model, during conditions of constitutive mTORC1 activation, IL-4 signaling through the JAK-STAT6 pathway is intact, however mTORC1-mediated attenuation of the parallel Akt pathway leads to impaired M2 polarization. Reciprocally, $Tsc1^{\Delta/\Delta}$ macrophages stimulated with proinflammatory stimuli such as LPS, are able to activate canonical inflammatory signaling pathways such as NF-kB, but due to attenuated Akt have exaggerated pro-inflammatory responses. As discussed above, the role of mTORC1 in M1 macrophages is unclear, and previous studies were conducted using pharmacological inhibitors of mTORC1. We show that short-term rapamycin treatment relieves negative feedback loops and enhances Akt activation; conversely, long-term rapamycin treatment leads to dissociation of the mTORC2 complex and can inhibit activation of Akt by blocking phosphorylation of Akt at serine 473 [13]. Thus, rapamycin's inability to inhibit the activity of mTORC1 without affecting the activity of Akt

and mTORC2 leads to confounding results that are hard to interpret. However, even genetic

approaches have yielded conflicting results. For example, in a study by Pan et al., deletion of

TSC1 in BMDMs showed pro-inflammatory effects, while Weichhart et al., showed that

knockdown of TSC2 had anti-inflammatory effects[9, 14]. The decreased inflammatory

responses in the Weichhart et al., study may be due to differences in cell type and/or use of

shRNA knockdown. Nonetheless, our data and those from Pan et al., show that TSC1-null

macrophages have a heightened pro-inflammatory phenotype. In Pan et al., they attribute this

pro-inflammatory phenotype to the ability of constitutively activated mTORC1 to activate JNK1/2

signaling. Since previous publications have shown JNK can phosphorylate IRS1/2 [15, 16], it is

tempting to speculate that upregulated JNK1/2 may partially contribute to the attenuated Akt

phosphorylation observed in $Tsc1^{\Delta/\Delta}$ macrophages. Thus, their findings offer another potential

mechanism for mTORC1 dependent feedback inhibition of Akt. In another study, Zhu et al. show

that mice with myeloid specific deletion of TSC1 also have attenuated Akt signaling leading to

defective M2 polarization and enhanced expression of M1 genes[17]. According to this study,

C/EBPβ, an Akt regulated transcription factor known to control expression of M2 genes, is also

downregulated in a mTORC1 dependent manner, while exaggerated M1 responses are

mTORC1 independent and largely reliant on an unknown role of TSC1 in promoting the MAPK

pathway[17]. Collectively, the studies in Chapter 2 and from other groups demonstrate the

importance of the mTOR pathway in the regulation of macrophage polarization and also

highlight the complexity and difficulty of studying this pathway due to negative feedback

mechanisms and signaling cross talk. Future studies examining the role of constitutive activation

of mTORC1 and its consequential actions on other signaling pathways such as Akt, mTORC2,

MAPK and JNK will be needed to further clarify the consequences of chronic activation of

mTOR at the molecular level and how mTOR affects macrophage activation.

The physiological consequences of constitutive mTORC1 in macrophages

Collectively, our study and other recent publications have shown that chronic activation of the mTORC1 pathway inhibits the ability of macrophages to respond appropriately to both pro and anti-inflammatory stimuli. Since macrophages are found in every organ and tissue and play an important role as frontline defenders against invading pathogens and in tissue homeostasis, we postulated that the inability of macrophages from $Tsc1^{\Delta/\Delta}$ mice to properly polarize to the alternative M2 state and their hyper-inflammatory phenotype would lead to inflammatory disorders and pathophysiological outcomes. Indeed, we observed that $Tsc1^{\Delta/\Delta}$ mice had hydrocephaly, suffered from seizures, displayed autistic-like behavior and were susceptible to premature death around 8-12 weeks of age. Therefore, we posit that this phenotype is consistent with a defect in the nervous system potentially due to hyper-inflamed microglia cells, resident macrophages found in the brain. Additionally, the Zhu et al. study showed that $Tsc1^{\Delta/\Delta}$ mice had high levels of immune cell infiltrates in the colon, lungs, and liver consistent with systemic inflammation and died from intestinal hemorrhaging [17]. Thus, chronic activation of mTORC1 in the innate immune system leads to profound inflammation and autoimmune disease with damage to multiple organs and can be fatal in mice.

In conditions of nutrient excess, increased production of insulin and pro-inflammatory cytokines found in metabolic tissues promotes elevated activation of the mTORC1 pathway, leading to S6K1 mediated negative feedback inhibition of insulin receptor substrate (IRS1/2) and insulin resistance [18]. Thus, to better understand the role of chronic activation of mTORC1 in a more physiological setting we investigated mTORC1 activation in the adipose tissue macrophage (ATM) containing stromal vascular fraction (SVF) isolated from the perigonadal adipose tissue of wild type (WT) mice in the setting of obesity, which contains roughly 40% F4/80+ macrophages[19]. Metabolic diseases, such as obesity and type-2 diabetes, are associated with chronic low-grade inflammation or "meta-inflammation", due to dysregulation of

metabolic and inflammatory signaling pathways[20]. During obesity monocytes are recruited to adipose tissue and polarize to an M1 state, displacing the M2 macrophages normally found in non-obese metabolic tissues[19]. M1 adipose tissue macrophages produce high levels of pro-inflammatory cytokines, which contribute to the development of Type-2 diabetes [21, 22]. In our study, mice on a high fat diet gained significantly more weight than mice fed a normal chow and the SVF of mice placed on a high fat diet had increased expression of inflammatory cytokines and decreased expression of M2 genes, suggesting a switch from M2 state to the M1 state during obesity (Figure 4-1A and 4-1B). We also observed increased mTORC1 activation as indicated by significant phosphorylation of S6 and concomitant decrease in phosphorylation of Akt^{T308} a critical site for Akt activation (Figure 4-1C and 4-1D**)**. Based on these results, we believe attenuated activation of Akt in ATMs during obesity is due to increased mTORC1 activity, as suggested by our evidence in Chapter 2. Thus, attenuated Akt in ATMs during obesity may be a potential mechanism in the polarization of macrophages from a M2 to M1 state and lead to enhanced expression of inflammatory cytokines, which exacerbates insulin resistance.

Since macrophages in $Tsc1^{\Delta/\Delta}$ mice have a similar phenotype to those in obese WT mice, we posited that these mice would be more susceptible to insulin resistance on high fat diet. To test this hypothesis we placed a cohort of $Tsc1^{\Delta/\Delta}$ and $Tsc1^{fl/fl}$ control mice on high fat diet for 16 weeks. Unexpectedly, $Tsc1^{\Delta/\Delta}$ mice were protected from high fat diet induced weight gain despite equal food intake and showed better glucose metabolism based on their ability to clear glucose at a faster rate during a glucose tolerance test (Figure 4-2A and 4-2B). To control for any behavioral defects and developmental issues in $Tsc1^{\Delta/\Delta}$ mice and given that $Tsc1^{\Delta/\Delta}$ mice are difficult to breed and often die early in adulthood, we generated chimera mice by irradiating WT mice and reconstituting them with bone marrow from $Tsc1^{\Delta/\Delta}$ or $Tsc1^{fl/fl}$ control mice. Similar to conventional $Tsc1^{\Delta/\Delta}$ mice, chimera $Tsc1^{\Delta/\Delta}$ mice did not gain weight on 8-

weeks high fat diet (Figure 4-2C) and showed similar or more sensitive responses to glucose and insulin tolerance test, respectively (Figure 4-2D and 4-2E). Given the striking similarity between conventional $Tsc1^{\Delta/\Delta}$ and chimera $Tsc1^{\Delta/\Delta}$ mice on high fat diet, constitutive activation of mTORC1 in macrophages appears to protect mice from weight gain and metabolic disease, despite the $Tsc1^{\Delta/\Delta}$ macrophages expressing more inflammatory cytokines. Thus, constitutive mTORC1 activation in macrophages does not appear to be sufficient to drive metabolic disease and under some conditions may present some beneficial effects and/or may require additional inputs, or lead to pathological consequences after a longer period of time that extends beyond our experimental protocol. Possible caveats to our study include the use of a floxed Tsc1 deletion in the myeloid lineage by crossing mice to those expressing cre-recombinase using the lysozyme-M promoter, since lysozyme-M can also be expressed by other myeloid lineage cells such as dendritic cells and granulocytes [23]. Thus, constitutive mTORC1 in these cells, in addition to macrophages, may contribute to the protection of $Tsc1^{\Delta/\Delta}$ and chimera $Tsc1^{\Delta/\Delta}$ mice from the detrimental affects of high fat diet on glucose metabolism. Additionally, Tsc1 may also regulate other cellular processes and pathways independent of mTORC1, such as the MAPK pathway that may also affect macrophage function *in vivo* [17]. Lastly, although the $Tsc1^{\Delta/\Delta}$ model makes a great genetic tool to investigate the role of mTORC1 in the myeloid lineage *in vivo*, deleting Tsc1 uncouples the activation of mTORC1 from the PI3K-Akt pathway, and artificially and constitutively activates mTORC1 above levels that are physiologically possible and concomitantly disturbing multiple processes downstream of mTORC1. Thus, the $Tsc1^{\Delta/\Delta}$ model does not accurately reflect the elevated mTORC1 levels seen in adipose tissue macrophages during obesity (FIGURE 4-1). In fact most models of tissue specific deletion of Tsc1 in other metabolic tissues such as the muscle and liver are paradoxically protected from high fat diet [24, 25]. Thus, a deeper understanding how the mTORC1 pathway regulates metabolism and physiology will be an interesting area of research for future studies.
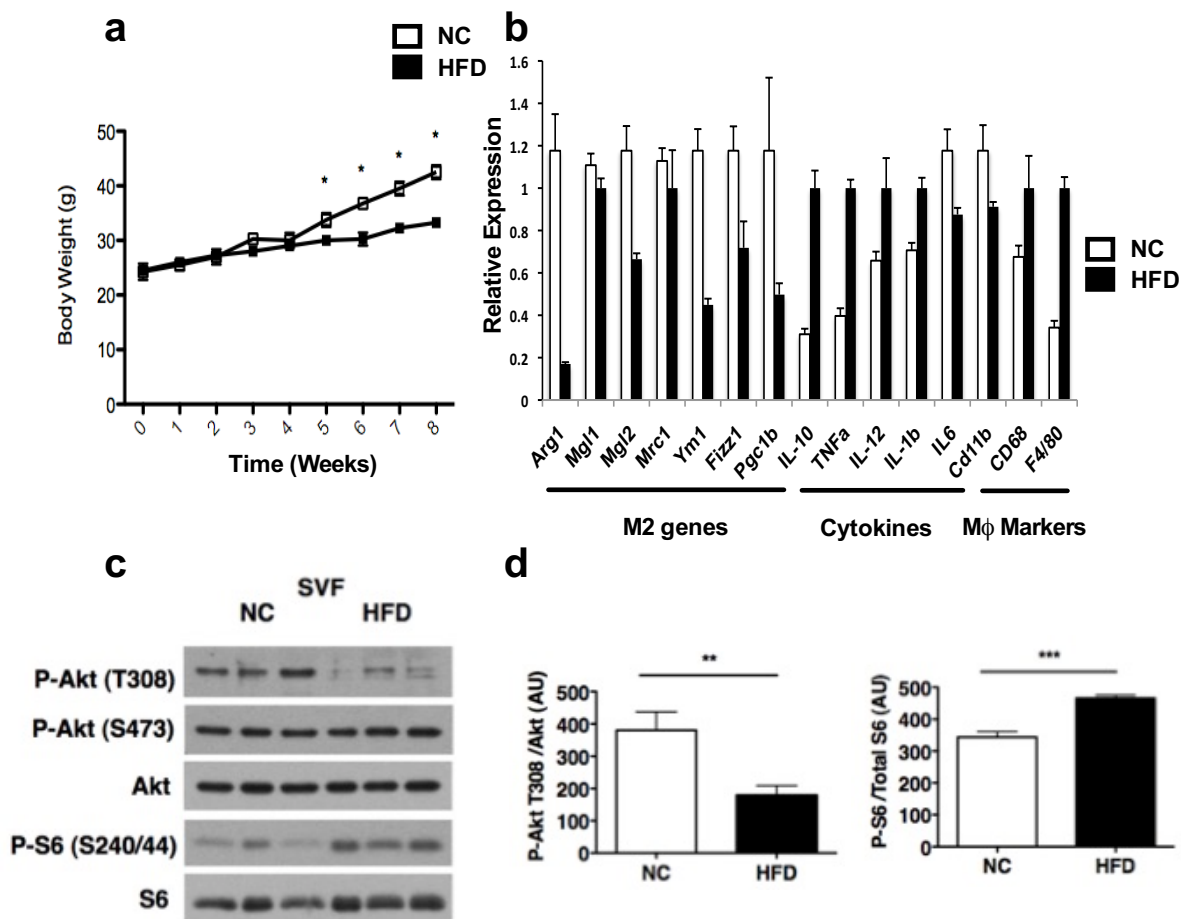
Figure 4-1. High fat diet leads to constitutive mTORC1 activation and attentuation of Akt in adipose tissue SVF.

A) Body weight of wild type male C57BL/6 mice fed a normal chow diet (NC) or high fat diet for 8 weeks (4 mice/group). (AC, VB)

B) M2 gene induction is reduced in HFD diet SVF. SVF was isolated from perigonadal adipose tissue after 8-weeks high fat diet and gene expression was measured by qPCR (average of 4 mice/group). (AC)

C) Immunoblot of SVF isolated from visceral fat of mice fasted 4 hours. Each lane represents one mouse. (AC)

Figure 4-1 (continued)

D) Quantitation of western blots in figure C using imagej software. (AC). The student's t-test was used to determine statistical significance, defined as *P<0.05, **P<0.01, and ***P<0.001.
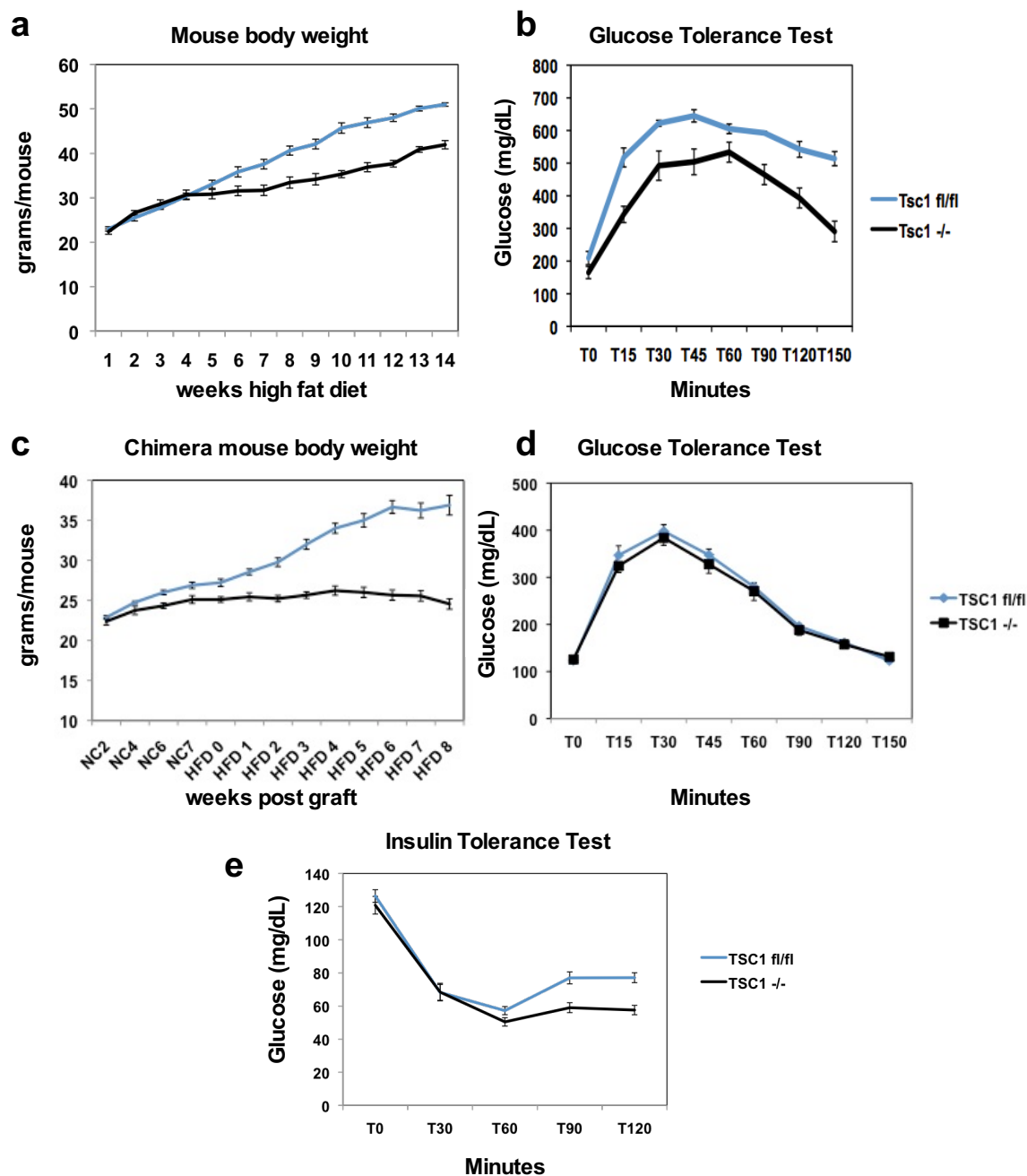
Figure 4-2. *Tsc1^{Δ/Δ}* mice are protected from weight gain and insulin resistance during high fat diet.

A) Body weight of male *Tsc1^{Δ/Δ}* and *Tsc1^{fl/fl}* control mice fed a high fat diet for 14 weeks (4 mice/group). (AC, JY)

Figure 4-2 (continued)

B) Glucose tolerance test in $Tsc1^{\Delta/\Delta}$ and $Tsc1^{fl/fl}$ mice fasted for 12 hours prior to being injected

intraperitoneally with 2g glucose/Kg body weight (results are mean +/- SEM, 4 mice/group).

(AC, JY)

C) Body weight of chimera mice (WT male mice with donor bone marrow from $Tsc1^{\Delta/\Delta}$ and

$Tsc1^{fl/fl}$ control mice) fed a high fat diet for 8 weeks (4 mice/group). (AC, JY)

D) Glucose tolerance test in chimera $Tsc1^{\Delta/\Delta}$ and $Tsc1^{fl/fl}$ mice fasted for 12 hours prior to being

injected intraperitoneally with 2g glucose/Kg body weight. Serum glucose levels were measured

for the indicated times (results are mean +/- SEM, 4 mice/group). (AC, JY)

E) Insulin tolerance test in chimera $Tsc1^{\Delta/\Delta}$ and $Tsc1^{fl/fl}$ mice fasted for 12 hours prior to being

injected intraperitoneally with 0.5 Units Insulin/Kg body weight. Serum glucose levels were

measured for the indicated times (results are mean +/- SEM, 4 mice/group). (AC, JY)

The role of acute activation of the Akt-mTORC1 pathway in IL-4 dependent M2 macrophage polarization

M2 polarization was first observed by stimulating macrophages with IL-4 leading to decreased oxidative burst, reduced expression of the inflammatory cytokine TNF-α, and increased expression of macrophage mannose receptor (Mrc1/2) [26-28]. Recently other markers for M2 macrophages have been identified such as Arg1, Mgl1, Fizz1 and Ym1[29]. IL-4 has been shown to induce gene expression in myeloid cells by signaling predominantly through the Type 1 IL-4 receptor α/γ chain and activating the JAK3-STAT6 pathway, leading to increased expression and activation of the nuclear receptors PPARγ and PPARδ. Activation of the nuclear receptors PPARγ and PPARδ in macrophages by Th2 cytokines has been shown to promote alternative activation via the regulation of metabolic genes that support beta-oxidation and oxidative phosphorylation [30, 31]. Additionally, STAT6 can also physically interact with PPARγ, as well as other IL-4 inducible transcription factors such as PGC1β and the histone acetyl-transferases CBP and P300, to modulate M2 gene expression [32-34]. In addition to STAT6, IL-4 activation of the Akt-mTORC1 pathway appears to be hard wired [10]. Thus, the work in chapter 2 only highlights the consequences of constitutive mTORC1, due to deletion of Tsc1, on M1 and M2 polarization, and does not address the role of acute/physiological activation of the mTORC1 pathway or the mechanism in which Akt promotes macrophage polarization.

In Chapter 3, our findings show that IL-4 promotes the acute activation of the PI3K-Akt pathway leading to phosphorylation of serine 455 of the enzyme ATP-Citrate Lyase (Acly), the primary enzyme responsible for producing nuclear-cytosolic acetyl-CoA [35]. Acly produces acetyl-CoA by cleaving mitochondrial-derived citrate to acetyl-CoA and oxaloacetate and its enzymatic activity is enhanced by Akt dependent phosphorylation at serine 455[36]. Furthermore, we show that inhibition of Akt or Acly with pharmacological inhibitors lead to

reduced acetyl-CoA levels and a significant reduction in the gene expression of a subset of M2 genes such as Arginase1, Fizz1 and Mgl2. A pivotal study by Wellen et al., showed that global histone acetylation and the expression of metabolic genes could be fine tuned by nuclear-cytosolic acetyl-CoA levels that were dependent on Acly, and coupled to metabolic input[37]. Similarly, we observed that IL-4 treatment of macrophages leads to an increase in the global acetylation of the histones H3 and H4 and at the promoters of Akt inhibitor sensitive genes but not Akt insensitive genes. Furthermore, IL-4 inducible histone acetylation could be repressed by blocking the Akt-Acly pathway and unbiased RNA-sequencing showed a significant overlap of Akt sensitive M2 genes and Acly sensitive M2 genes, including chemokines and genes involved in cellular proliferation.

Downstream of the Akt-Acly pathway, we show that the histone acetyl-transferase P300 partly mediates gene specific acetylation of Akt sensitive gene promoters and has no affect on the acetylation of the promoters of Akt insensitive genes, despite P300's ubiquitous presence at most macrophage enhancers [38]. Macrophage gene expression is regulated by both lineage determining transcription factors such as PU.1 and stimulus regulated transcription factors such as NF-kB and STAT6, for macrophages stimulated with LPS and IL-4 respectively. Lineage determining transcription factors act as pioneering factors which in concert with histone methyltransferases and histone acetyl transferases modify histone tails with post-translational modifications that include methylation of H3K4 and acetylation of H3K27 to facilitate the accessibility of the macrophage genome to transcription factors, including those that are stimulus dependent [39]. Although PU.1 and P300 are constitutively bound to active and poised macrophage specific enhancers, only stimuli specific enhancers belonging to genes induced by stimuli such as LPS or IL-4 have inducible P300 binding[38]. Thus, a limitation of our RNA-Seq data is it does not allow us to see the IL-4 induced transcription factors that regulate inducible P300 binding to the promoters and enhancers of IL-4 dependent genes. Therefore, future

studies including ChIP-Seq using antibodies for P300, STAT6, and different histone marks such as H3K27 will be necessary to determine if Akt-sensitive IL-4 inducible genes has significantly greater inducible P300 binding and if acetylation of these enhancers is affected by Akt inhibition. Once this data is obtained we can use a bioinformatic approach to scan promoters motifs of IL-4 inducible genes and determine if we can identify transcription factors enriched for Akt sensitive genes versus Akt insensitive genes. Each putative transcription factor candidate can then be further studied using tools such as Crispr/Cas9 to delete them and determine how it may regulate IL-4 inducible gene induction.

In addition to histone acetylation, other groups have reported that IL-4 treatment of macrophages leads to the activation of the histone demethylase JMJD3 to control M2 polarization by promoting the demethylation of the IRF4 promoter, thereby promoting IRF4 expression and the expression of IRF4-dependent M2 genes [40, 41]. Thus, it would be of great interest to investigate not only IL-4 inducible histone acetylation marks but also histone methylation marks to better understand how metabolic input and the Akt pathway regulates chromatin remodeling and M2 gene expression at a global level.

Direct regulation of M2 polarization by MTORC1

During activation of immune cells, metabolic shifts occur to meet the energetically costly demands of immune responses. For example, it has been shown that LPS treated M1 macrophages rely primarily on glycolytic metabolism, known as Warburg metabolism, while M2 macrophages metabolize glucose, glutamine, and fatty acids to support oxidative phosphorylation [32, 42, 43]. In our study, we also show that IL-4 treatment of macrophages leads to increased glycolytic and oxidative phosphorylation and uptake of glucose, glutamine, and fatty acids to fuel anabolic processes such as acetyl-CoA production (Figure 4-3) and presumably other anabolic processes such as nucleotide and protein synthesis. Since anabolic

processes are energetically demanding, we believe the Akt-mTORC1 pathway is able to act as a licensing checkpoint that is activated only when nutrients are abundant and growth conditions are favorable. In support of this we showed in Chapter 3 that increasing amino acid levels could directly activate the PI3K-Akt/mTORC1-Acly pathway, where activation of mTORC1 drives increased protein levels of Acly, while concomitant Akt activation by amino acids promotes Acly phosphorylation, thereby controlling acetyl-CoA production and gene expression of Akt/Acly dependent M2 genes. Furthermore, adipose tissue macrophage containing SVF from mice in the fed state also showed increased Akt activation and histone acetylation and had higher expression of M2 markers compared to their fasting counterparts. Taken together, our data suggest that the Akt-Acly pathway couples nutrient sensing and metabolic input to coordinate M2 gene expression in a gene specific manner *in vitro* and *in vivo* via Akt dependent control of Acly production of acetyl-CoA to control histone acetylation of a subset of M2 genes. In addition to nutrient levels, hormones including insulin can also activate the Akt-mTORC1 pathway, thus future studies looking at the role of insulin in macrophages will also be interesting, given that although macrophages express the insulin receptor, the effect of insulin on macrophage function remains undefined[44].

Our work in Chapter 2 shows that constitutive activation of the mTORC1 pathway alters macrophage polarization by promoting feedback mechanisms which attenuate activation of Akt leading to reduced expression of M2 genes and increased expression of inflammatory genes. However, IL-4 acutely activates mTORC1 within minutes and may drive M2 polarization by promoting anabolic metabolism and transcriptional regulation of enzymes involved in metabolic shifts, such as glycolysis and oxidative phosphorylation[45]. Therefore, to test the role of acute mTORC1 in the regulation of M2 polarization we deleted Raptor in macrophages (referred to as $Raptor^{\Delta/\Delta}$ BMDMs or $Raptor^{fl/fl}$ control BMDMs) by adding tamoxifen to BMDMs from mice with floxed alleles of the Raptor gene and expressing the estrogen receptor fused to cre-
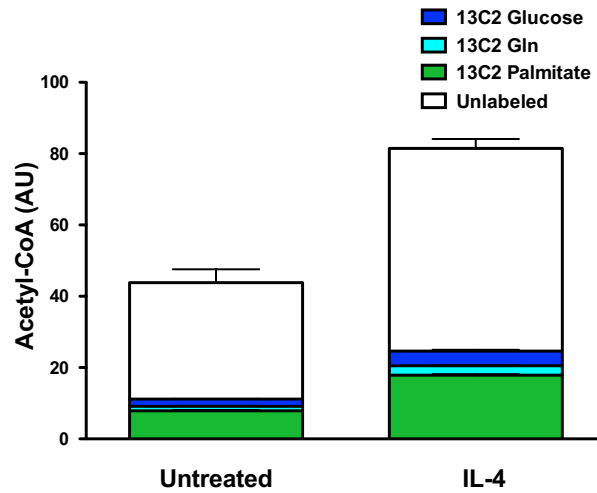
Figure 4-3. Glucose, Glutamine and Fatty acids are carbon sources for IL-4 inducible Acetyl-CoA. BMDMs were stimulated for 10h with IL-4, followed by a 2h incubation with $^{13}C6$-glucose, $^{13}C6$-glutamine or $^{13}C16$-palmitate. Carbon tracing into acetyl-CoA was determined by detection of ^{13}C(M+2) acetyl-CoA by mass spec. (AC, NS, AW)

recombinase. As expected, deletion of Raptor leads to significant loss of mTORC1 activity as demonstrated by decreased phosphorylation of the mTORC1 target S6K in $Raptor^{\Delta/\Delta}$ BMDMs (Figure 4-4A). Interestingly, $Raptor^{\Delta/\Delta}$ BMDMs had significantly reduced expression of Akt/Acly sensitive genes such as Arg1, Fizz1, and Mgl2, while having no effect on Akt/Acly insensitive genes (Figure 4-4B). To my knowledge, this is the first direct evidence of mTORC1 to directly promote M2 polarization. Furthermore, this data reinforces our hypothesis that a subset of M2 genes is sensitive to nutrient sensing pathways and metabolic input. However, additional studies such as RNA-Seq will need to be performed to determine how much overlap there is between Raptor sensitive genes and Akt/Acly sensitive genes. Since, deletion of Raptor leads to decreased protein levels of Acly (Figure 4-4A), perhaps some of the reduction in Akt/Acly sensitive genes may be due to decreased production of Acetyl-CoA. Additionally, mTORC1 has also been shown to regulate Acly transcript levels via SREBP[46], however we do not see a difference in Acly mRNA levels in $Raptor^{\Delta/\Delta}$ BMDMs versus control BMDMs (AJC observation). Thus, mTORC1 may regulate Acly translation/protein levels independent of SREBP in M2 macrophages. Given the pleiotropic functions of mTORC1 in cell biology, it is very likely that mTORC1 can also regulate multiple downstream targets and pathways independent of Akt/Acly to promote M2 gene expression. Thus, additional studies will be needed to determine the many potential mechanisms by which mTORC1 promotes macrophage activation and polarization.

Macrophages can directly sense amino acids levels via the PI3K-Akt pathway to control mTORC1 and mTORC2 activation

Amino acids are critical regulators of mTORC1 activity and activate the RAG family of small GTPases, which recruit mTORC1 via a complex termed the Ragulator to the lysosomal surface, which is both necessary and sufficient for activation of mTORC1 [47]. While the mechanisms in which amino acids are sensed have remained elusive, recent papers suggest
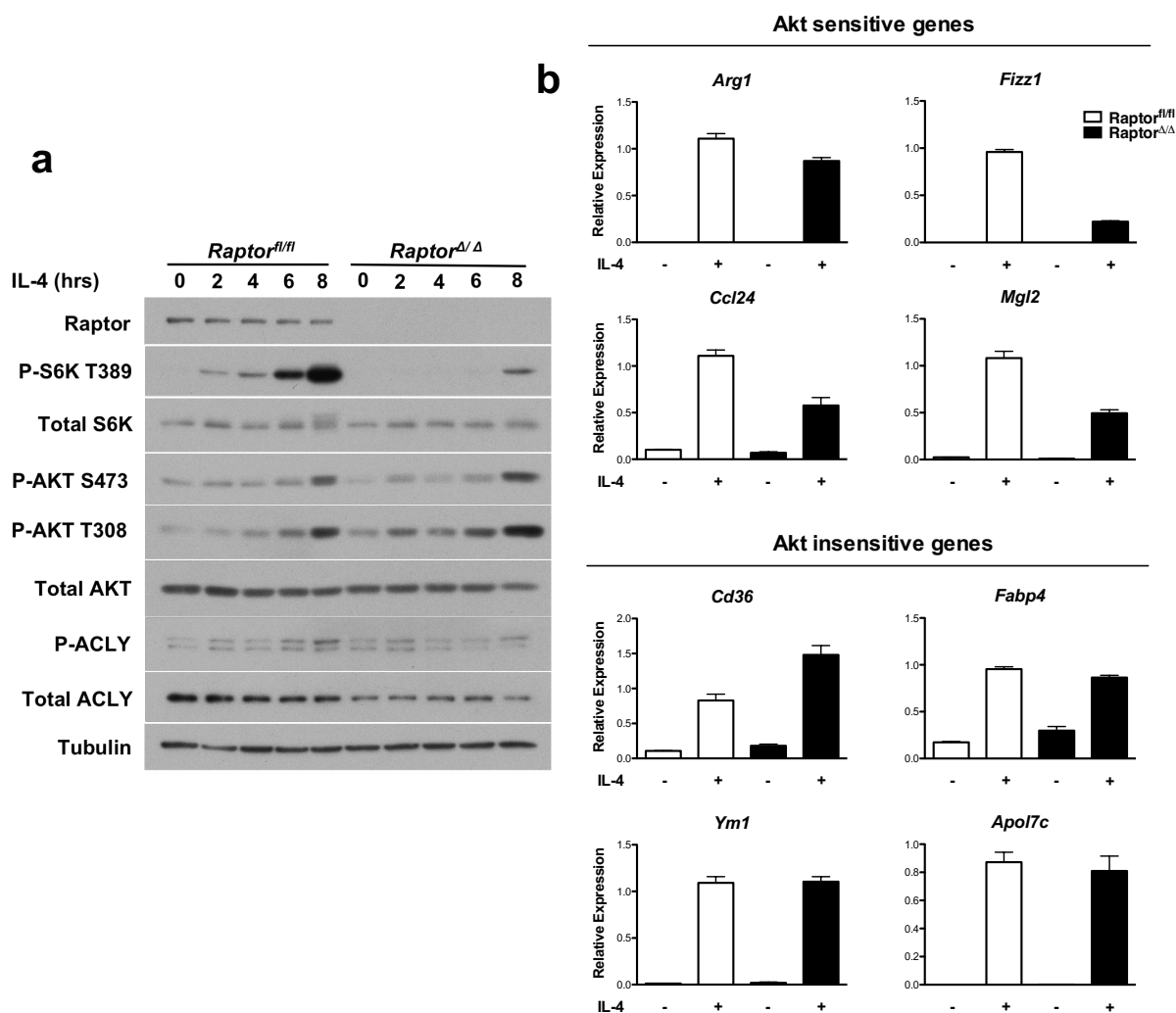
Figure 4-4. Acute activation of mTORC1 by IL-4 regulates a subset of M2 genes.

A). Deletion of Raptor decreases mTORC1 activation. Immunoblot of *Raptor$^{Δ/Δ}$* BMDMs and *Raptor$^{fl/fl}$* control BMDMs treated with IL-4 for the indicated times. (AC)

B) mTORC1 regulates gene expression of Akt sensitive M2 genes. BMDMs stimulated as indicated with IL-4 for 16 hours and gene expression measured by RT-PCR. Fold change was calculated by making the control WT IL-4 treated sample 1 and the fold change of other samples are calculated relative to this sample. (AC)

the SLC38A9 lysosomal amino acid transporter and Sestrin2 can directly sense amino acids

such as arginine/glutamine and leucine, respectively, and activate the RAG proteins to control

mTORC1 activity [48-50]. In parallel to amino acids, growth factors activate mTORC1 via a

protein complex called tuberous sclerosis complex (TSC). This complex contains multiple

subunits including TSC1, TSC2, and TBC1D7 and has GAP activity negatively regulating the

activation of the small GTPase Rheb by keeping it in a GDP bound form [51]. Conversely, the

GTP-bound form of Rheb critically activates mTORC1 at the lysosomal surface via an unknown

mechanism. Growth factors activate the PI3K-Akt pathway, which inactivates the TSC complex,

relieving its inhibition of Rheb and allowing for mTORC1 activation. Our data in chapter 3 shows

that amino acids levels and leucine can critically regulate the Akt/mTORC1-Acly pathway and

are important metabolites for alternative macrophage metabolism and effector function.

Interestingly, preliminary data in our lab shows that amino acids can directly activate mTORC1

as indicated by phosphorylation of S6K, and mTORC2 as indicated by phosphorylation of

NDRG1, and is dependent on activation of the PI3K -Akt pathway since treatment with the PI3K

inhibitor LY294002 abrogates these affects (Figure 4-5). To our knowledge this is the first

evidence of the ability of amino acids to activate mTORC1 via the PI3K pathway, while the

ability of amino acids to activate mTORC2 via PI3K has only been observed by one other group

[52]. This data suggest that macrophages have the unique ability to directly sense amino acid

levels by an unknown/novel mechanism that is dependent on PI3K production of PIP3 and hints

at the possibility that macrophages may express a cell surface membrane receptor that can

sense amino acid levels. Nonetheless, additional studies will be necessary to reveal how

macrophages can directly sense amino acids to activate mTORC1 and mTORC2 in the absence

of growth factors and the physiological relevance of this finding.
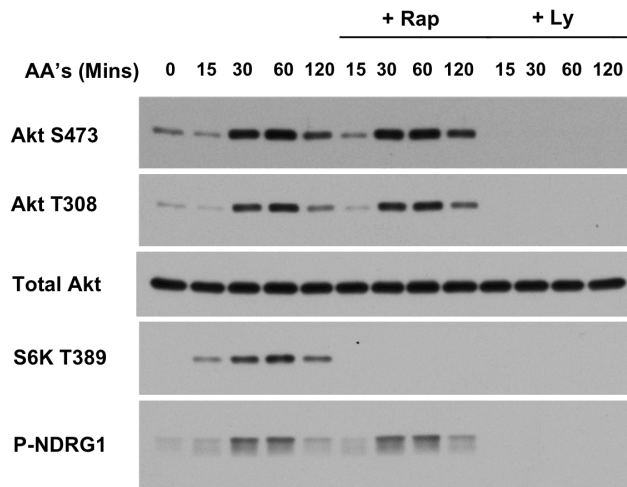
Figure 4-5. Macrophages can directly sense amino acids to activate the mTORC1 and mTORC2 pathway. Immunoblot of BMDMs serum starved for 6 hours prior to being acutely starved of amino acids for 45 minutes and/or treated with the PI3K inhibitor LY294002 (Ly) or Rapamycin (Rap) and re-stimulated with media containing amino acids for the indicated period of time. (AC, IBS)

Future Directions

The Akt/mTORC1-Acly pathway couples metabolic input to control a subset of M2 genes via gene specific histone acetylation. Thus, as a future direction we would like to determine what metabolic pathways and carbon sources contribute to the production of cytosolic acetyl-CoA during IL-4 activation. In a recent study, Jha et al. showed via isotopic carbon and nitrogen tracing that IL-4 treated macrophages utilize glycolytic and glutamine metabolism as the major carbon and nitrogen sources, respectively, for production of the hexosamine pathway metabolite UDP-GlcNAc which is used for N-glycosylation of M2 genes such as the mannose receptor (CD206)[43]. Given that M2 polarization leads to increased glycolysis, beta-oxidation, and oxidative phosphorylation, we would like to use a similar approach and use isotopic carbon tracing of C13 labeled glucose, glutamine, or fatty acids to determine which fuel substrate contributes to the cytosolic acetyl-CoA pools used to acetylate the histones located on the promoters of Akt/Acly inhibitor sensitive genes. Additionally, some carbon substrates may be utilized for other anabolic purposes such as the hexosamine pathway discussed above for protein N-glycosylation, the oxidative or non-oxidative pentose phosphate pathway for nucleotide synthesis, and for anaplerosis to replenish TCA cycle intermediates. Thus, combining metabolomic flux experiments with RNA-seq and signaling studies will better clarify how the Akt-mTORC1 pathway coordinates metabolic reprogramming to effector functions that occur in response to both pro and anti-inflammatory stimuli.

Macrophages play an important role in many physiological settings such as fighting pathogens and maintaining metabolic health. Thus, we believe the Akt/mTORC1-Acly pathway is important for the proper function of tissue resident and recruited macrophages *in vivo*, particularly in metabolic tissues since the Akt-mTORC1 pathway fine-tunes immune response to metabolic input. Based on our RNA-seq data and gene ontology analysis, the Akt/mTORC1-

Acly pathway regulates the expression of chemokines, therefore we posit that this pathway, in addition to regulating macrophage polarization, acts as a metabolic checkpoint to coordinate the recruitment of other immune cells to Th2 driven inflammatory sites. In support of this hypothesis, a study by Sinclair et al. showed that lymphocyte trafficking is regulated by the PI3K-Akt-mTORC1 pathway to synchronize immune responses to metabolism[53]. Thus, to test this hypothesis we have bred mice with Akt1 and Akt2 floxed alleles, the predominant isoforms of Akt expressed in macrophages, to mice expressing cre-recombinase using the lysozyme promoter to delete Akt1, Akt2, and both Akt1 and Akt2 in myeloid cells. We hypothesize that mice with deletion of Akt1 and/or Akt2 will be protected from Th2 inflammation and therefore we will test this by utilizing an ovalbumin (Ova)-induced asthma model. In future studies we will also investigate other disease models in which M2 macrophages play a role in such as in obesity (mentioned above) and cancer where there is a growing appreciation of the role M2-like tumor associated macrophages (TAMs) play in promoting tumorigenesis. We believe the intersection of signaling and metabolism in macrophages is rife with discoveries that will unlock interesting biological findings that will be important and relevant for better understanding and development of cures for metabolic syndrome, autoimmune diseases, cancer, and pathogen associated diseases that affect human health.

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Appendix

Ros sets the stage for macrophage for differentiation

Adapted from:

Anthony J. Covarrubias[1], Vanessa Byles[1] and Tiffany Horng[1]. (2013). Ros sets the stage for macrophage for differentiation. Cell Research. 23(8): 984-5.

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Summary

While M1 macrophages are highly pro-inflammatory and microbicidal, M2 macrophages and the related tumor associated macrophages (TAMs) regulate tissue remodeling and angiogenesis and can display immunomodulatory activity. Here Zhang et al show that ROS production—critical for the activation and functions of M1 macrophages—is necessary for the differentiation of M2 macrophages and TAMs, and that antioxidant therapy blocks TAM differentiation and tumorigenesis in mouse models of cancer.

Discussion

Macrophages are key orchestrators in both the initiation and resolution stages of inflammation, and function as sentinel cells that maintain homeostasis and protect against infection. They are activated by many stimuli including pathogen-associated molecular patterns (PAMPs), endogenous danger-associated molecular patterns (DAMPs), and cytokines found in the tissue microenvironment[1]. During their activation, macrophages can polarize to pro-inflammatory or anti-inflammatory states with distinct phenotypes and physiological responses—the classical pro-inflammatory M1 state induced by LPS and Interferon-γ and the "alternative" M2 state triggered by IL-4 and IL-13[2]. The M1 state is characterized by increased expression of pro-inflammatory cytokines as well as microbicidal activity, while M2 macrophages upregulate the anti-inflammatory cytokine IL-10 and participate in tissue remodeling, wound repair, and host defense against large parasites.

M2-like macrophage polarization is of particular pathophysiological consequence in the setting of cancer. Early in tumor development, monocytes are recruited by tumor and stromal cell-derived chemokines to take up residence at the tumor site, where they differentiate into macrophages in response to MCSF produced by tumor cells. Such tumor-associated macrophages (TAMs) facilitate multiple steps in tumorigenesis, including promotion of tumor cell

proliferation and resistance to apoptosis as well as secretion of pro-angiogenic factors and proteolytic enzymes that aid in tumor cell metastasis. TAMs also display some immunosuppressive features, such as IL-10 and TGF-β production and poor antigen presentation, which conspire to prevent tumor cell killing by infiltrating T cells. Thus, the characteristics most critical for the tumor-promoting profile of TAMs bear semblance to the M2 phenotype. Although the details of such M2 polarization are not well characterized, IL-4 produced by T-cells in the tumor, as well as other tumor-derived factors, may be critical[3].

In this issue of Cell Research, a study by Zhang et al provides new insights into control of macrophage differentiation and activation. In particular, the authors show that ROS production is important in M2 but not M1 macrophage differentiation (Figure A-1). Their experimental protocol is to treat monocytes for 6 days with M-CSF or GM-CSF to induce differentiation to macrophages, followed by polarization with IL-4 (M2 state) or LPS and IFN-γ (M1 state). Interestingly, pre-treating monocytes with the antioxidant butylated hydroxyanisole (BHA) prior to differentiation inhibits M2 but not M1 polarization, as indicated by analysis of macrophage differentiation markers and M1/M2 polarization markers. The authors attribute this to the effects of BHA—i.e. block of ROS production—in inhibiting ERK activation during macrophage differentiation, consistent with previous reports implicating a role for ROS as well as MAP kinases in macrophage differentiation[4]. Furthermore, LPS and IFN-γ but not IL-4 stimulation can "rescue" ERK activation—perhaps in a manner dependent upon ROS-production—thus explaining why M2 but not M1 polarization is impaired by antioxidant treatment.

Because the M2-like properties of TAMs are though to promote tumorigenesis, Zhang et al go on to investigate the consequences of BHA administration in mouse models of cancer. They demonstrate that in vivo treatment of BHA can attenuate cancer initiation, progression, and metastasis in multiple models. Because ROS can promote tumor cell proliferation, survival, and DNA damage, BHA could be acting directly on the tumor cells to prevent growth and

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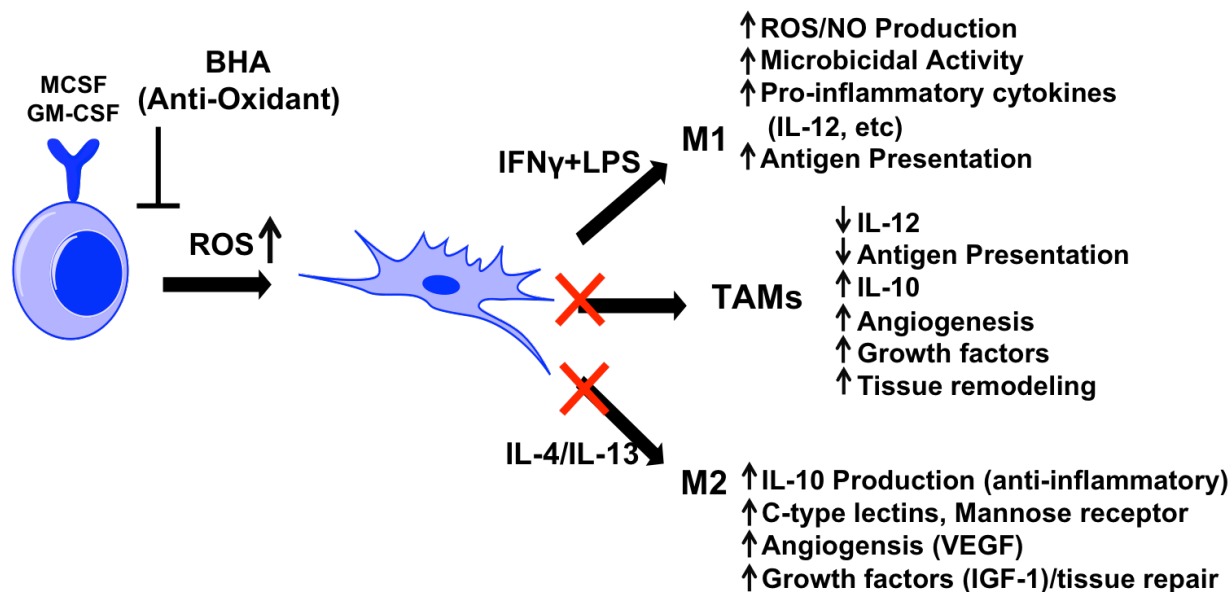
Figure A-1. M1 macrophages are highly pro-inflammatory and microbicidal and are polarized by treatment with LPS+IFNγ, while M2 macrophages mediate tissue repair, angiogenesis and immunomodulation. Tumor associated macrophages (TAMs), which are M2-like, are associated with worsened clinical prognosis in many cancers and are thought to be skewed by a combination of tumor-derived factors and other cytokines present in the tumor microenvironment. ROS production increases during M-CSF or GM-CSF-induced macrophage differentiation from monocytes, and the anti-oxidant BHA specifically inhibits M2 and TAM polarization. LPS+IFNγ treatment is able to overcome the effects of BHA to induce normal M1 polarization, revealing a specific role for ROS in macrophage polarization.

metastasis[5]. However, BHA had no effects on the proliferation of three tumor cell lines in vitro. Instead, the authors propose that TAM differentiation may be a critical target, since BHA administration reduced TAM numbers as well as levels of TAM markers. Moreover, in at least in one of the models, BHA administration was ineffective when macrophages were depleted by clodronate injection.

Collectively, the findings of Zhang et al are intriguing for several reasons. First, ROS production is usually associated with the activation and functions of M1 rather than M2 macrophages. ROS production downstream of LPS signaling mediates production of pro-inflammatory cytokines (in part through MAP kinase activation). ROS and nitric oxide (NO) production by NADPH oxidase and iNOS respectively as well as mROS upregulation are key to the antimicrobial activity of M1 macrophages[6]. Indeed NO production can inhibit oxidative metabolism, pivotal to the survival and function of M2 macrophages[7]. Thus ROS production may be important in M1 activation and function while the requirement for ROS in M2 differentiation may be most critical during MCSF-mediated differentiation rather than IL-4-triggered polarization. Future studies to better understand the role of ROS production in macrophage differentiation and activation may be informative. Second, it would be interesting to further probe the effects of BHA in inhibiting tumorigenesis. The authors' in vitro studies suggest inhibition of TAM differentiation as one underlying mechanism, but one can envision additional possibilities. At least in some cancers, tumor cells and other immune cells in the microenvironment produce ROS that promote inflammation[8], thus contributing to tumorigenesis. mROS has been linked to activation of HIF1a, which can facilitate angiogenesis and metastasis. Indeed, it is worth pointing out that ROS can regulate many cellular processes, some of which have already been eluded to, including signal transduction (e.g. downstream of growth factor receptors and innate immune signaling pathways as well as MAP kinase activation); redox-signaling; autophagy; and respiratory burst and other antimicrobial activities[9].

Thus it is likely that other cellular processes perturbed by antioxidant treatment contribute to the effects of BHA in reducing tumorigenesis.

Finally, this study from Zhang et al suggests that treatment with BHA or perhaps other antioxidants could be considered in therapeutic control of cancer. Indeed, there is tremendous interest in the clinical use of anti-oxidants for treating many diseases. Given the pleiotropic activities of ROS mentioned above, it would be important to better understand the molecular pathways by which anti-oxidants exert their effects.

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Appendix

IL-6 strikes a balance in metabolic inflammation

Adapted from:

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Summary

IL-6 is a pleiotropic cytokine that exerts either proinflammatory or anti-inflammatory effects, and is implicated in diverse settings including obesity, exercise, arthritis, and colitis. A new study by Mauer et al shows that modulation of macrophage activation by IL-6 maintains glucose homeostasis in diet-induced obesity while limiting inflammation in endotoxemia.

Discussion

IL-6 is generally considered a pro-inflammatory cytokine. It promotes neutrophilia and TH17 differentiation while blocking Treg differentiation, and critically contributes to pathophysiology in rheumatoid arthritis, EAE, and other inflammatory diseases. However, there is some evidence to suggest an immunomodulatory role for IL-6 in certain contexts. Genetic deficiency of IL-6 increases inflammatory responses to local and systemic endotoxin administration, while muscle-derived IL-6 may mediate some of the anti-inflammatory and insulin-sensitizing effects of physical exercise. Thus, available evidence is consistent with pleiotropic functions for the cytokine[1]. Interestingly, how IL-6 regulates macrophage biology remains not well understood, even though the effects of IL-10, which signals through a similar pathway, has long served as a paradigm for immunomodulation of macrophage activation. In a new study, Mauer et al make the unexpected and important finding that IL-6 can control macrophage activation. IL-6 potentiates alternative or M2 macrophage activation by IL-4, resulting in improved metabolic responses to high fat diet challenge, but attenuates classical or M1 macrophage activation to LPS, thus conferring protection to endotoxemia[2].

Mauer et al. first addressed a role of IL-6 in controlling macrophage activation in the context of metabolic homeostasis, where the modality of macrophage activation is known to be critical. M2 activation of adipose tissue macrophages, mediated by IL-4 and/or IL-13 production from eosinophils and/or ILC2 cells, favors insulin sensitivity, while M1 activation, triggered by

saturated fatty acids and/or adipocyte derived inflammatory cytokines, contributes to obesity-associated chronic inflammation (or metaflammation), insulin resistance, and systemic metabolic deterioration[3, 4]. The authors found that upon challenge with high fat diet, mice with myeloid specific deletion of IL-6Ra (*IL6ra*^Dmyel^) were more glucose intolerant and insulin resistant despite comparable food intake and weight gain. This may be a result of increased metaflammation in white adipose tissue, brown adipose tissue and liver, as indicated by increased expression of markers of M1 macrophages, while expression of M2 markers was reduced. Therefore, IL-6 may modulate macrophage activation during diet-induced obesity to attenuate inflammatory responses and resist metabolic dysregulation.

To address a potential role of IL-6 in controlling macrophage activation, Mauer et al first challenged mice with myeloid specific deletion of IL-6Ra (*IL6ra*^Dmyel^) to diet-induced obesity[2]. Previous studies indicate that the modality of macrophage activation is critical in this context. M2 activation of adipose tissue macrophages, mediated by IL-4 and/or IL-13 production from eosinophils and/or ILC2 cells, favors insulin sensitivity, while M1 activation, triggered by saturated fatty acids and/or adipocyte derived inflammatory cytokines, contributes to obesity-associated chronic inflammation (or metaflammation), insulin resistance, and systemic metabolic deterioration[3, 4]. The authors found that despite comparable food intake and weight gain, *IL6ra*^Dmyel^ mice were more glucose intolerant and insulin resistant on a high fat diet. This may be a result of increased metaflammation in white adipose tissue, brown adipose tissue and liver, as indicated by increased expression of markers of M1 macrophages, while expression of M2 markers was reduced. Therefore, IL-6 may modulate macrophage activation during diet-induced obesity to attenuate inflammatory responses and resist metabolic dysregulation.

To elucidate the basis for such modulation of macrophage activation, the authors used microarray analysis to identify IL-6-regulated responses[2]. Interestingly, *IL-4ra* emerged as one of the most strongly inducible genes in IL-6-stimulated bone marrow derived macrophages

(BMDMs), and appeared to be a direct target of Stat3, the major transcription factor activated by IL-6 signaling. Accordingly, IL-6 increased IL-4-mediated induction of multiple M2 genes *in vitro* and *in vivo*. In addition, *IL-4ra* expression in WAT was reduced in obese *IL6ra*^Dmyel^ mice, which dovetails nicely with the relative inability of exogenous IL-4 to improve glucose tolerance in these mice.

Conversely, Mauer et al showed that IL-6 exerts opposing effects on M1 activation, since pretreatment of BMDMs with IL-6 attenuated LPS-mediated induction of multiple genes[2]. Indeed, *IL6ra*^Dmyel^ mice were more susceptible to endotoxemia, as indicated by increased plasma levels of proinflammatory cytokines, and exacerbated weight loss and reduced food intake. Collectively, these findings establish a role for IL-6 in promoting M2 activation but attenuating M1 activation (Figure A-2).

The findings in this study suggest that in the obese state, increased IL-6 production[5] may be an adaptive response to metabolic stress. Because IL-6 is made by many cell types and tissues and is released into the circulation, the potential sources are manifold and could include adipocytes and the liver. Such IL-6 production potentiates M2 activation of adipose tissue macrophages by IL-4 and presumably IL-13 (which also signals through the IL-4Ra). In parallel, IL-6 may dampen M1 activation by saturated fatty acids and LPS, and perhaps other inflammatory cytokines, all of which are elevated in obesity. Thus IL-6 may be produced as a negative feedback mechanism during chronic nutrient excess to tune macrophage activation (Figure A-2). The complex and nuanced functions of IL-6 may explain the conflicting literature on its role in metabolic regulation, with some studies supporting beneficial effects while others indicating detrimental activities[5]. For example, IL-6-deficient mice develop mature onset obesity in one study but not another, while acute infusion of IL-6 can impair insulin sensitivity or enhance glucose tolerance. Of note, the emerging view of IL-6 as both an immunoregulatory and pro-inflammatory cytokine is consistent with its dual roles in catabolic (e.g. increasing
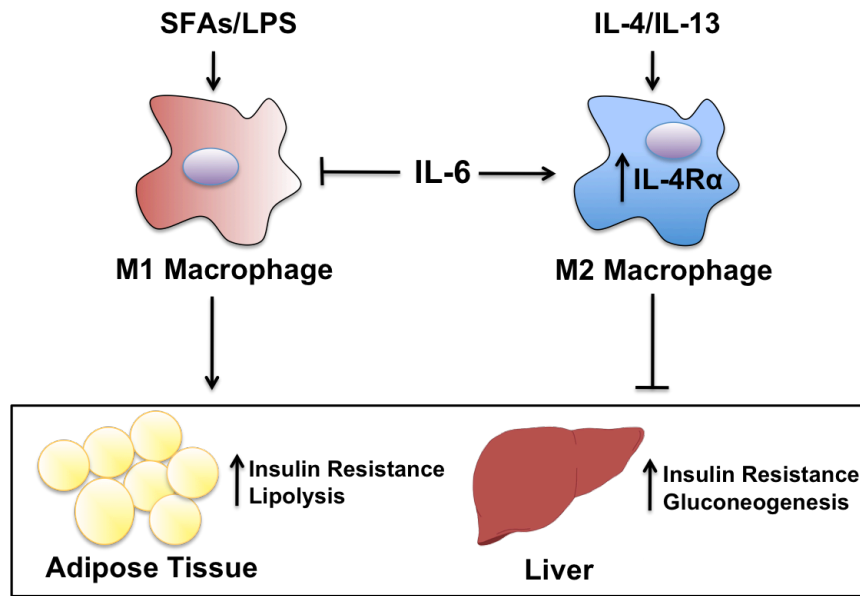
Figure A-2. IL-6 Limits Obesity-Induced Insulin Resistance by Promoting Macrophage M2 Polarization. In the obese state, increased production of IL-6 promotes M2 activation of adipose tissue macrophages by upregulating the expression of IL-4ra. In parallel, IL-6 may inhibit the effects of saturated fatty acids (SFAs) and lipopolysaccharide (LPS) in triggering M1 activation. Thus, IL-6 regulates activation of adipose tissue macrophages to attenuate inflammatory responses, improve insulin sensitivity, and resist metabolic dysregulation.

lipolysis) and anabolic (e.g. increasing insulin sensitivity) metabolism. While the complexities of IL-6 function will take more time to sort out, one intriguing possibility is that its source, target and circulating levels can determine its pro-inflammatory versus immunomodulatory effects. In this regard, it has been proposed that IL-6 signaling via the classical pathway (through IL-6Ra and gp130) and in *trans* (by binding of a IL-6 and soluble IL-6Ra complex to cell surface gp130) may lead to different outcomes[1].

The study raises other interesting points for discussion. While IL-4Ra upregulation would undoubtedly sensitize macrophages to IL-4, IL-6 is likely to promote M2 activation via additional mechanisms, for example via AMPK and/or PI3K/Akt activation which are implicated in macrophage polarization to a M2 or anti-inflammatory phenotype. Such mechanisms could allow for modulation of specific subsets of M2 genes, as opposed to *IL-4ra* induction, which would presumably increase induction of all M2 genes. Regardless of the underlying mechanism, knowing whether IL-6 synergizes in the induction of a subset or the entirety of the M2 program may offer additional insights into the rationale of M2 activation by IL-6. How IL-6 negatively regulates M1 activation was not addressed and would also be important to explore. Finally, does IL-6 regulate M2 activation in other physiological and pathophysiological contexts? Exercise, cold stress, and fasting have all been shown to increase circulating levels of IL-6, and M2 activation in these contexts may promote muscle regeneration, adaptive thermogenesis, and lipid scavenging, respectively[6-10]. Related to this, IL-6 can stimulate whole body and intramyocellular fatty acid oxidation as well as lipolysis[5]. Since beta-oxidation supports M2 activation (as opposed to glycolytic metabolism in the case of M1 macrophages)[3], such effects of IL-6 would enable coordination of M2 activation with the systemic metabolic profile.

Despite its discovery nearly 30 years ago, how IL-6 controls macrophage biology has remained poorly understood. The study by Mauer et al highlights the role of IL-6 as an

important regulator of macrophage activation, and is sure to spark new interest in this

fascinating cytokine.

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