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The role of mammalian target of rapamycin (mTOR) in macrophage polarization

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THE ROLE OF MAMMALIAN TARGET OF RAPAMYCIN (mTOR) IN
MACROPHAGE POLARIZATION

by

VANESSA A. BYLES

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Approved by

First Reader

____________________________________________________________________

Susan K. Fried, Ph.D.
Professor of Medicine

Second Reader

____________________________________________________________________

Tiffany Horng, Ph.D.
Assistant Professor of Genetics and Complex Diseases
Harvard School of Public Health
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THE ROLE OF MAMMALIAN TARGET OF RAPAMYCIN (mTOR) IN MACROPHAGE POLARIZATION

VANESSA A. BYLES

Boston University School of Medicine, 2013

Major Professor: Susan K. Fried, Ph.D., Professor of Medicine

ABSTRACT

Macrophages are key orchestrators of the innate immune response with a dynamic role in the promotion and resolution of inflammation. Macrophage polarization to a pro-inflammatory or anti-inflammatory phenotype must be tightly controlled to maintain appropriate responses to stimuli as well as to maintain tissue homeostasis. The nutrient and energy sensor Mammalian Target of Rapamycin (mTOR) integrates upstream signals from the PI3K/Akt pathway to orchestrate cellular protein, lipid, and glucose metabolism. This key metabolic pathway has been implicated in T-helper cell skewing and in the innate immune regulation. The mechanisms of innate immune regulation by mTOR are currently unclear as most studies use pharmacological inhibitors with potential off target effects. In this study, we use a novel model of TSC1 deficiency in myeloid lineage cells to elucidate a role for mTOR in macrophage polarization. We show, for the first time, that Tsc1-deficiency and constitutive mTORC1 activity in macrophages leads to a marked defect in M2 polarization when stimulated with the Th2 cytokine IL-4. Tsc1-deficient macrophages display attenuated Akt signaling in response to IL-4 consistent with negative feedback of mTORC1 on upstream IRS2/PI3K signaling, and we demonstrate that this parallel signaling pathway is critical for induction of a subset of M2 markers. Tsc1-deficient macrophages fail to upregulate...
the M2 genes \( Pgc-1\beta, Arg-1, Fizz-1, \) and \( Mgl1 \) in addition to other M2 markers despite normal STAT6 signaling in response to IL-4. Consistent with downregulation of \( Pgc-1\beta \), \( Tsc1 \)-deficient macrophages also display defects in fatty acid metabolism and mitochondrial biogenesis. Furthermore, LPS stimulation in \( Tsc-1 \) deficient macrophages leads to an enhanced inflammatory response with increased production of pro-inflammatory cytokines. We believe that \( Tsc1 \)-deficient macrophages are a model of constitutive mTORC1 activity akin to obesity, where chronic nutrient excess leads to increases in mTORC1 activity, attenuation of IRS/PI3K/Akt signaling, and defective M2 polarization of macrophages in metabolic tissues.
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Akt Regulates a Subset of M2 Macrophage Markers

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ABBREVIATIONS

BMDMs  Bone Marrow Derived Macrophages
FOXO1  Forkhead Box Transcription Factor 1
GSK3   Glycogen Synthase Kinase-3
IL-4   Interleukin-4
MEF    Mouse Embryonic Fibroblasts
mTOR   Mammalian Target of Rapamycin
mTORC  mTOR Complex (1&2)
PPARγ/δ Peroxisome Proliferator-Activated Receptor gamma, delta
PGC-1β  Peroxisome Proliferator-Activated Receptor gamma Coactivator
PIP    Phosphatidylinositol Phosphate2
TLR4   Toll-Like Receptor 4
TNF-α  Tumor Necrosis Factor- alpha
TSC    Tuberous Sclerosis Complex
Introduction

Mammalian target of rapamycin (mTOR) is a key nutrient and energy sensor that integrates cell surface receptor signals and amino acid availability to regulate cell growth, proliferation and survival. mTOR, a serine/threonine kinase, exists in two complexes in the cell; mTORC1 containing rapamycin-sensitive Raptor and mTORC2 containing rapamycin-insensitive Rictor, both of which are positively by insulin, cytokines and growth factors. mTOR Complex 1 regulates protein synthesis and ribosomal biogenesis through phosphorylation of downstream targets S6K1/2 and the translational repressor 4E-BP1. A recent study also demonstrates that mTORC1 promotes glycolytic metabolism as well as lipid and cholesterol biosynthesis through regulation of HIF-1α and SREBP1/2, respectively. mTORC2 function, on the other hand is not well understood. The tuberous sclerosis repressor complex containing TSC1 and TSC2, integrates upstream signals of nutrient and energy availability to regulate mTORC1 and mTORC2 activity. The TSC complex possesses GTPase Activating Protein activity, which exerts effects on the small GTPase protein Rho. In the absence of nutrient and growth factor signaling, the TSC complex negatively regulates mTORC1 activity by maintaining Rho in the GDP-bound, inactive state. Conversely, in the presence of nutrients and growth factors, PI3K-mediated Akt activation leads to phosphorylation and inactivation of TSC2 GAP activity allowing Rho to become GTP-bound and activate mTORC1. Mutations in the TSC complex thus lead to aberrant mTORC1 activity, which is notably linked to the tuberous sclerosis genetic disorder, increased cancer cell proliferation, and insulin resistance. Mouse models of Tsc1 or Tsc2 genetic ablation are increasingly utilized to elucidate the function of mTORC1 in several cell types and tissues both in vitro and in vivo.
A key event that occurs upstream of mTORC1 activation is phosphorylation of the serine/threonine kinase Akt. PI3K-mediated PIP2 to PIP3 conversion downstream of the insulin receptor and other cytokine and growth factor receptors activates PDK1, which phosphorylates Akt at threonine 308, whereas PI3K activation mTORC2 leads to Akt phosphorylation at serine 473. Downstream, Akt inactivates the TSC repressor complex in addition to phosphorylating the transcription factor, FOXO1, leading to nuclear exclusion. Constitutive mTORC1 in Tsc1 or Tsc2-null cells results in negative feedback inhibition of upstream Akt signaling. This negative feedback inhibition, likely a mechanism to maintain inducibility of these signaling pathways, occurs by two defined mechanisms; first, an increase in S6K1 activity downstream of mTORC1 in Tsc-null cells leads to serine phosphorylation of insulin receptor substrates (IRS1/2), which destabilizes these proteins and attenuates downstream insulin-stimulated Akt phosphorylation. Second, loss of the TSC complex in addition to direct inhibition of S6K1 leads to reduced mTORC2-mediated Akt phosphorylation at serine 473, in addition to decreased activity of other mTORC2 targets. Thus, loss of either Tsc1 or Tsc2 leads to a decrease in inducible Akt phosphorylation and activity due to constitutive mTORC1 resulting in insulin resistance, and this is partially reversed by the mTORC1 inhibitor rapamycin in hepatocytes, MEFs, and adipocytes.

Several studies now demonstrate that mTOR is a central regulator of key metabolic processes in a number of cells types, but the role of mTOR in regulating immune cell function is just beginning to emerge. A recent study, for example, elucidated a role for mTOR kinase in the regulation of T-helper cell function. The study identified that mTORC1 was essential for differentiation of naive T-cells into pro-inflammatory Th1 cells, while mTORC2 was essential for differentiation into anti-inflammatory Th2 cells.
Another study in dendritic cells, a component of the innate immune system, reported that knockdowns of Tsc2 leads to decreased pro-inflammatory cytokine secretion with bacterial stimulation, while mTORC1 inhibition with rapamycin leads to enhanced pro-inflammatory responses\(^9\). While there is evidence of mTOR regulating both the innate and adaptive immune system in T-helper cells and dendritic cells, the role of mTOR in regulating macrophage cell function and metabolism remains poorly understood.

Macrophages represent a key component of the innate immune system responsible for orchestrating the inflammatory response and host defense\(^10\). Circulating monocytes derived from the bone marrow are differentiated into macrophages in tissues by cytokines or growth factors that polarize macrophages into pro-inflammatory or anti-inflammatory phenotypes\(^10\). Macrophages activated by lipopolysaccharide, a component of bacteria, and Interferon-\(\gamma\) are pro-inflammatory and are referred to as “classically activated,” or M1\(^10\). M1 macrophages promote tissue damage and inflammation by producing cytokines, such as TNF-\(\alpha\), IL-6 and IL-1\(\beta\), chemokines, such as MCP-1, and lipid mediators, such as prostaglandins and leukotrienes\(^10\). Additionally, M1 macrophages dramatically increase iNOS mRNA expression and activity, which generates nitric oxide from L-arginine substrate to promote bacterial killing\(^10,11\).

Conversely, macrophages activated by Interleukin-4 are anti-inflammatory and are referred to as “alternatively activated,” or M2\(^12\). M2 macrophages promote the resolution of inflammation and tissue repair by producing factors such as Fizz1, C-type lectins Mgl1/2, Mrc1, and Ym1\(^13\). A hallmark of M2 macrophages is an increase in Arginase-1 expression and activity, which utilizes L-Arginine as a substrate to generate L-ornithine, an amino acid used for polyamine synthesis, and the byproduct urea\(^12,14,15\). In addition to
orchestrating the immune response, circulating monocytes and resident macrophages are critical for maintaining tissue homeostasis\textsuperscript{16, 17}. The pivotal role of these immune cells thus necessitates a careful balance between pro-inflammatory M1 and anti-inflammatory M2 function\textsuperscript{16, 17}. If the balance of M1 and M2 macrophages is disrupted, this could lead to inappropriate responses to environmental stimuli and exacerbated pathology in certain settings\textsuperscript{16}. In the pathological context of obesity, for example, adipose tissue macrophages undergo a phenotypic switch from M2 to M1, which potentiates inflammation and associated insulin resistance\textsuperscript{18}.

Not only do M1 and M2 macrophages have divergent and opposing functions, they also have distinct metabolic programs to meet cellular energy demands during activation. Alternatively activated M2 macrophages utilize fatty acid oxidation mediated by the nuclear receptors PPAR\(_\delta\) and PPAR\(_\gamma\), while classically activated M1 macrophages primarily rely on glycolytic metabolism, mediated by HIF-1\(\alpha\)\textsuperscript{14, 19}.

Particularly in M2 macrophages, regulation of the metabolic program and expression of M2 markers necessary for alternative activation are intricately linked. For instance, the fatty acid sensors peroxisome proliferator activated receptors \(\gamma\) and \(\delta\) (PPAR\(_\gamma\) and PPAR\(_\delta\)) promote fatty acid oxidation and M2 gene marker expression in conjunction with the coactivator PGC-1\(\beta\)\textsuperscript{14}. Two notable studies demonstrate that loss of either PPAR\(_\gamma\) or PPAR\(_\delta\) in myeloid lineage cells leads to a defect in M2 polarization\textsuperscript{20, 21}. Specifically, \(PPAR\delta\)-KO macrophages have reduced expression of a subset of M2 genes, such as the C-type lectin family members \(Mgl1\) and \(Mgl2\), as well as \(Arginase-1\) upon IL-13 stimulation\textsuperscript{21}. \(PPAR\gamma\)-KO macrophages have defective Arginase-1 expression and activity in addition to marked defects in fatty oxidation upon IL-4 stimulation\textsuperscript{20}. The PPAR coactivator, PGC-1\(\beta\), is also critical for M2 differentiation, with PGC-1\(\beta\) knockdown in
macrophages leading to decreased Arginase-1 activity and expression concomitant with defects in fatty acid oxidation. In addition to PPARs, the transcription factor STAT6 is indispensable for M2 polarization. STAT6 is phosphorylated by Janus Kinase-1 downstream of IL-4 receptor signaling, which leads to dimerization and nuclear translocation. STAT6 then increases expression of several M2 genes and Pgc-1β, as well as genes involved in oxidative metabolism. PGC-1β serves as a transcriptional coactivator for STAT6 as well as PPARδ, and PPARγ, which promotes the metabolic shift to oxidative metabolism and the full expression of M2 markers. In addition to STAT6 and PPAR activation, IL-4 Receptor signaling in macrophages leads to activation of IRS2 and downstream PI3K/Akt signaling. The IL-4Rα chain specifically engages IRS2 in response to IL-4 leading to downstream Akt activation, an event shown to be critical for induction of the M2 gene IL-1R antagonist. Proper Akt signaling downstream of the IL-4 Receptor in parallel with JAK/STAT6 signaling and PPAR activation may be important for maximal expression of M2 markers and implementation of metabolic programs in M2 polarization.

In this study, we use Tsc1-deficient macrophages to investigate the role of mTOR in macrophage polarization. We show, for the first time, that Tsc1-deficiency and constitutive mTORC1 activity in macrophages leads to a marked defect in M2 polarization when stimulated with the Th2 cytokine IL-4. Tsc1-deficient macrophages display attenuated Akt signaling in response to IL-4 consistent with negative feedback of mTORC1 on upstream IRS2/PI3K signaling, and we demonstrate that this parallel signaling pathway is critical for induction of a subset of M2 markers. Tsc1-deficient macrophages fail to upregulate the M2 genes Pgc-1β, Arg-1, Fizz-1, and Mgl1 in addition to other M2 markers despite normal STAT6 signaling in response to IL-4.
Consistent with downregulation of Pgc-1β, Tsc1-deficient macrophages also display defects in fatty acid metabolism and mitochondrial biogenesis. Furthermore, LPS stimulation in Tsc-1 deficient macrophages leads to an enhanced inflammatory response with increased production of pro-inflammatory cytokines. We believe that Tsc1-deficient macrophages are a model of constitutive mTORC1 activity akin to obesity, where chronic nutrient excess leads to increases in mTORC1 activity, attenuation of IRS/PI3K/Akt signaling, and defective M2 polarization of macrophages in metabolic tissues.

**Materials and Methods**

**Mice**

Mice with floxed Tsc1 alleles (Tsc1^fl/fl^) were kindly provided by Brendan Manning (Harvard School of Public Health) and LysozymeM-Cre transgenic mice were kindly provided by Chihao Lee (Harvard School of Public Health). Rictor^fl/fl^ and Rictor^fl/fl^ ubiquitinC-Cre ERT2 mice injected with tamoxifen were kindly provided by David Sabatini. All mice were on a B6 background and were described previously. For targeted deletion of Tsc1 in myeloid lineage cells, Tsc1^fl/fl^ mice were crossed to LysMCre transgenic mice. Genotype of the mice was confirmed by PCR of tail DNA with primers to detect the Tsc1 loxP alleles and the LysMCre transgene. All mice were maintained at Harvard Medical School, and procedures were performed in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee.

**Bone Marrow Derived Macrophages**

Bone marrow derived macrophages were prepared as previously described. Briefly, femurs were removed from mice after euthanasia with CO₂, and cells were subsequently liberated from the femur bones using a mortar and pestle. For macrophage
differentiation, bone marrow derived cells were plated in petri dishes with complete 1640 RPMI media (10% FCS, Penicillin/Streptomycin, 2mM L-Glutamine) supplemented with MCSF-containing L929 cell supernatant for seven days. After seven days, MCSF differentiated macrophages were harvested with PBS + 2mM EDTA and plated in tissue culture dishes for subsequent experiments. For M1 polarization, 0.5x10^6 were plated in 12-well tissue culture dishes and treated with 10ng/ml LPS (Invivogen). For M2 polarization, cells were treated with 10ng/ml IL-4 (Peprotech).

Reagents and Antibodies

To examine mTORC1 signaling in primary macrophages, antibodies to phospho-S6K1 (Thr389), phospho-4E-BP1 (Ser65), phospho-S6 (Ser40/44), total S6K1, total S6 and total 4E-BP1 were used. Antibodies to phospho-Akt (Thr308, Ser473), pan-Akt, phospho-Foxo1 (Thr24), Total Foxo1, phospho-GSK3α/β (Ser21/9), Total GSK3α/β, Total IRS2, phospho-STAT6 (Tyr641), total STAT6, and β-actin were also used. All antibodies were from Cell Signaling Technology. mTORC1 and Akt signaling antibodies and Aktvii inhibitor (EMD) were generously provided by Dr. Brendan Manning (Harvard School of Public Health). STAT6 antibodies and Troglitazone (Cayman) were generously provided by Dr. Chih-Hao Lee (Harvard School of Public Health). α-Tubulin antibody was purchased from Sigma Aldrich and rapamycin was purchased from LC Laboratories.

Immunoblotting, qPCR, and ELISA

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 (β-glycerophosphate, sodium fluoride, sodium orthovanadate). Protein concentration in lysates was determined using the Bradford method. For immunoblotting, 15µg of protein was loaded onto SDS-PAGE gels.
Gels were subsequently transferred onto PVDF membranes. Membranes were then blotted with primary antibodies as indicated. After primary antibody incubation, membranes were incubated with anti-rabbit or anti-mouse HRP secondary antibodies (GE Healthcare). Western blot membranes were developed using Femto or Pico ECL reagent (Thermo Fisher Scientific). NIH ImageJ Software was used for densitometry.

For RNA isolation, cells were collected in RNA-Bee (Tel-Test Inc.) followed by chloroform extraction and isopropanol precipitation according to the manufacturer’s instructions. RNA pellets were washed twice with 75% ethanol, dried and resuspended in water. RNA (1µg) was used for cDNA synthesis using the Applied Biosystems cDNA synthesis kit according to the manufacturer’s instructions. qPCR analysis was performed using Bio-Rad Sybr green reagent and the Bio-Rad Real-Time system. Transcript levels were normalized to HPRT and relative expression was plotted using the ΔΔCt method.

To measure cytokine secretion by ELISA, cell culture supernatants were collected and debris was spun out by centrifugation at 3000xg, 5 min. Supernatants were diluted in assay diluent for TNF-α ELISAs while supes were used undiluted for IL-10 ELISA. All ELISA kits were purchased from BioLegend, and assays were performed according to the manufacturer’s instructions.

**Arginase Assays**

Arginase assay was described previously. Briefly, 0.5x10^6 cells/well in 12-well plates were stimulated with 10ng/ml IL-4 for 12-48h. Cells were lysed in 0.1% TritonX-100 lysis buffer. Lysates were incubated with 500mM of L-Arginine for 45 minutes at 37°C, and the reaction was subsequently stopped with acid solution. The degradation of L-arginine to urea was measured by adding 9% isonitrosopropiophenone in 100%
ethanol and reading absorbance at 540nm in a microplate reader. All samples were read in triplicate.

**Fatty Acid Oxidation and Mitochondrial Mass Measurements**

Macrophages were plated 0.7x10^6 cells/well in 12-well tissue culture dishes in complete RPMI and stimulated with IL-4 for 24-36h. After stimulation, cells were washed with PBS and loaded with low glucose DMEM plus 2% fatty acid-free BSA for 30 minutes at 37°C. After 30 minutes, cells were washed twice with PBS and given ³H-labeled palmitic acid (2µCi/well) in low glucose DMEM plus 2% fatty acid-free BSA, plus 0.2mM unlabeled palmitic acid. After 4h, 100ul of media was collected and the isolation of ³H₂O was performed using trichloroacetic acid followed by chloroform-methanol extraction. Water-soluble fractions were collected into 5ml of EcoLume 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 ³H was subtracted from the CPM value and all samples were normalized to mg of total protein. For mitochondrial mass, cells were plated 0.7x10^6 cells/well in low binding 12-well plates and stimulated with 10ng/ml IL-4 for 24h. After 24h, cells were stained with 25nM MitoTracker® Green (Invitrogren) for 30 min. Cells were then harvested in FACS buffer (PBS+2mM EDTA+0.5% FCS) and analyzed by flow cytometry using a BD FACSCalibur machine. All data was analyzed using FlowJo software (TreeStar).

**Results**

**Confirmation of Tsc1fl/fl and Tsc1fl/fl LysMCre Mouse Genotype**

TSC1 (hamartin) is part of the tuberous sclerosis repressor complex that in partnership with TSC2 (tuberin) GAP activity regulates the Rheb, the small G-protein
that activates mTORC1\textsuperscript{3}. To study mTORC1 function in macrophages, we used a gain a function approach generating mice with myeloid specific deletion of \textit{Tsc1}. We took advantage of the Cre-lox system and crossed \textit{Tsc1}\textsuperscript{loxP/loxP} mice with mice carrying transgenic Cre driven by the myeloid specific gene promoter, \textit{Lysozyme M}, whose expression increases during macrophage differentiation\textsuperscript{26}. Cre recombinase activity leads to deletion of exons 17 and 18 of \textit{Tsc1} as previously described\textsuperscript{27}. To confirm the presence of the loxP alleles and the \textit{LysMC}re transgene, tail DNA was analyzed by PCR with primers to wild-type and loxP \textit{Tsc1} and to the \textit{LysMC}re transgene. Bone marrow derived macrophages from \textit{Tsc1}\textsuperscript{loxP/loxP} and \textit{Tsc1}\textsuperscript{loxP/loxP} \textit{LysMC}re mice, herein referred to as \textit{Tsc1}\textsuperscript{fl/fl} and \textit{Mac-Tsc1KO} respectively, were used in subsequent experiments. \textit{Tsc1} knockdown or deletion leads to constitutive mTORC1 activity independent of nutrients, growth factors and upstream PI3kinase/Akt signaling\textsuperscript{2}. To verify that \textit{Mac-Tsc1KO} bone marrow derived macrophages have constitutive mTORC1 activity, MCSF-differentiated BMDMs were incubated in the presence or absence of serum and the specific mTORC1 inhibitor, rapamycin. Western blotting shows that TSC1 is present in \textit{Tsc1}\textsuperscript{fl/fl} macrophages, but absent in the \textit{Mac-Tsc1KO} BMDMs, indicating that recombination was successful (Figure 1a). Since TSC1 stabilizes, TSC2, \textit{Mac-Tsc1KO} BMDMs also have diminished TSC2 protein (Figure 1a). Further western blot analysis examining mTORC1 downstream targets reveals that \textit{Mac-Tsc1KO} BMDMs have high basal phosphorylation S6K1 at threonine 389 and 4E-BP1 at serine 65 (Figure 1a). The mTORC1-specific inhibitor, rapamycin diminishes the constitutive phosphorylation S6K1 and 4E-BP1 in \textit{Mac-Tsc1KO} BMDMs (Figure 1a).
Inflammatory Phenotype of Mac-Tsc1KO BMDMs

Several studies have implicated mTORC1 in the modulation of immune function through the use of rapamycin, but the literature is quite conflicting due to off target effects of this drug. Studies using myeloid-specific genetic models to determine the role of mTOR in innate immune cells are lacking. In one recent study, investigators used a tamoxifen-inducible ubiquitin-cre system to delete Tsc1 in vivo, and found that bone marrow derived macrophages from mice injected with tamoxifen displayed enhanced secretion of pro-inflammatory cytokines IL-6 and TNF-α, which was attributed to increased JNK activity. Consistent with these findings, we show that upon LPS challenge that promotes an M1 phenotype, Mac-Tsc1KO BMDMs have enhanced secretion of the pro-inflammatory cytokine, TNF-α, and reduced secretion of the anti-inflammatory cytokine, Interleukin-10 by ELISA (Figure 1b). In addition, gene expression analysis reveals enhanced expression of pro-inflammatory cytokines TNF-α, IL-6, and IL-12p40 in Mac-Tsc1KO BMDMs when stimulated with LPS (data not shown). The increase in TNF-α secretion in Mac-Tsc1KO BMDMs was inhibited by rapamycin, indicating that constitutive mTORC1 promotes a pro-inflammatory phenotype (Figure 1c).

Mac-Tsc1KO BMDMs have Defective M2 Polarization

Given that Mac-Tsc1KO BMDMs display a pro-inflammatory phenotype, we hypothesized that there may be a defect in M2 polarization. When stimulated with the Th2 cytokine IL-4, Mac-Tsc1KO BMDMs display a marked failure to upregulate genes involved in M2 polarization. Mac-Tsc1KO BMDMs have a 2-3-fold reduction in Arginase-1, Mgl1, and Fizz1 mRNA expression relative to Tsc1fl/fl controls (Figure 2a). Consistent
with Arginase-1 gene expression, Arginase-1 activity indicated by urea production is 2-fold lower in Mac-Tsc1KO BMDMs relative to Tsc1fl/fl controls (Figure 2a). The defect in M2 gene expression in Mac-Tsc1KO BMDMs is not dose-dependent, as the defective induction of Arginase-1, Fizz1, and PGC-1β is present even when cells are stimulated with up to 100ng/ml IL-4 (data not shown). Given that the nuclear receptors PPARγ and PPARδ are known regulators of M2 polarization and that mTORC1 was shown to regulate PPARγ expression in adipose tissue, we analyzed gene expression of both PPARγ and PPARδ. We found that the mRNA expression levels of both Pparγ and Pparδ were unaffected in the Mac-Tsc1KO BMDMs at basal state and upon IL-4 stimulation (Figure 2b). Since PPAR gene expression levels were normal in the Mac-Tsc1KO BMDMs, we next wanted to check gene expression of Pgc-1β, the PPAR transcriptional coactivator also shown to be critical for M2 polarization. Indeed, mRNA levels of Pgc-1β were reduced approximately 2-fold in IL-4 stimulated Mac-Tsc1KO BMDMs relative to Tsc1fl/fl controls (Figure 2c). Consistent with the role of PGC-1β in promoting the fatty acid oxidation program of M2 macrophages, Mac-Tsc1KO BMDMs show impaired beta oxidation of palmitic acid, producing approximately 2-fold less 3H2O from labeled palmitic acid than Tsc1fl/fl controls (Figure 2c). Furthermore, mitochondrial biogenesis as measured by flow cytometry using MitoTracker® Green staining is modestly increased by IL-4 stimulation in Tsc1fl/fl BMDMs, while this increase is not observed in Mac-Tsc1KO BMDMs (Figure 2c). Stimulation of macrophages with IL-4 leads to IL-4 Receptor-dependent activation of JAK1, phosphorylation of STAT6 at tyrosine 641, dimerization and nuclear translocation12,14. When stimulated with IL-4 for 15min up to 2h, Mac-Tsc1KO BMDMs display normal, if not enhanced, phosphorylation of STAT6 at tyrosine 641, while total STAT6 levels were comparable between Mac-
Tsc1KO and Tsc1fl/fl BMDMs (Figure 2d). Furthermore, nuclear/cytosolic fractionation shows that phosphorylated STAT6 nuclear translocation was normal in Mac-Tsc1KO BMDMs, indicating that proper JAK1/STAT6 signaling is intact (Figure 2e). Taken together, these results indicate that constitutive mTORC1 in macrophages leads to defective M2 polarization despite normal PPAR expression and intact canonical IL-4 Receptor signaling.

Mac-Tsc1KO BMDMs have Diminished mTORC2 Activity and Attenuated Akt Signaling

Tsc1-null MEFs and hepatocytes both display attenuated insulin stimulated Akt signaling and reduced phosphorylation of downstream Akt targets. Another characteristic of Tsc1-null MEFs and hepatocytes that further impairs insulin signaling is reduced phosphorylation of IRS-1, which destabilizes the protein and reduces activation of PI3K/Akt signaling. Previous studies demonstrate that the IL-4 Receptor, particularly the IL-4Rα chain, engages IRS2 leading to tyrosine autophosphorylation, and downstream activation of the PI3K/Akt/mTOR pathway. We thus hypothesized that Mac-Tsc1KO BMDMs would have attenuated Akt in response to IL-4 stimulation similar to attenuated insulin signaling in other Tsc1-null cell lines. Indeed, western blot analysis shows that IL-4 stimulated Mac-Tsc1KO BMDMs display reduced phosphorylation of Akt at serine 473, the mTORC2 site, and at threonine 308, the PDK1 site (Figure 3a). Total Akt levels were comparable between Tsc1fl/fl and Mac-Tsc1KO Macrophages, although there is a downward mobility shift in Mac-Tsc1KO BMDMs (Figure 3a). Consistent with attenuated Akt signaling, phosphorylation of the Akt target FOXO1 is also reduced in the Mac-Tsc1KO BMDMs; however, phosphorylation of the Akt targets GSK3α/β is unaffected in Mac-Tsc1KO BMDMs (Figure 3a). GSK3α/β lies downstream of many signaling pathways, such as the Wnt and Ras pathways, which
could account for the normal phosphorylation levels\textsuperscript{29}. Total IRS2 levels were reduced in IL-4 stimulated \textit{Mac-Tsc1KO} BMDMs consistent with serine phosphorylation leading to protein destabilization and enhanced degradation (Figure 3a). Concomitant with attenuated Akt, \textit{Tsc1-null} MEFs also have diminished growth factor stimulated mTORC2 activity due to the mTORC1 negative feedback inhibition of upstream PI3K and loss of the tuberous sclerosis repressor complex necessary for mTORC2 activation\textsuperscript{3,30,31}. Following activation by PI3K, mTORC2 phosphorylates downstream targets SGK1 and PKC-\(\alpha\) in addition to Akt on serine 473\textsuperscript{31}. To assess mTORC2 activity in \textit{Mac-Tsc1KO} BMDMs, phosphorylation of NDRG1, a target of SGK1 was examined by western blot. Consistent with other \textit{Tsc1-null} cell types, IL-4 stimulated \textit{Mac-Tsc1KO} BMDMs display reduced phosphorylation of NDRG1 at threonine 346 relative to \textit{Tsc1\textsuperscript{fl/fl}} controls, indicating that mTORC2 activity is diminished (Figure 3a).

\textit{M2 Polarization is not defective in Rictor-null BMDMs}

To complement the \textit{Tsc1-deficiency} model that is characterized by attenuated mTORC2 and PI3K/Akt signaling, we took advantage of a tamoxifen-inducible ubiquitin\textit{C CreERT2} model to delete Rictor, the key component of mTORC2. Macrophages isolated from \textit{Rictor\textsuperscript{fl/fl}} and \textit{Rictor\textsuperscript{fl/fl Ub-Cre}} mice injected with tamoxifen were stimulated with IL-4 similar to the \textit{Mac-Tsc1KO} BMDMs. Western blot analysis shows that Rictor protein expression is completely ablated in the \textit{Rictor\textsuperscript{fl/fl Ub-Cre}} BMDMs indicating that cre-recombinase was successful (Figure 3b). Surprisingly, Arginase-1 activity is not reduced in the \textit{Rictor\textsuperscript{fl/fl Ub-Cre}} BMDMs, which is consistent with normal gene expression of \textit{Arginase-1} (Figure 3c). Furthermore, gene expression of other M2 markers, \textit{Pgc-1\(\beta\)}, \textit{Mgl1}, and \textit{Fizz1} is unaffected in IL-4 stimulated \textit{Rictor-null} BMDMs (Figure 3c and data not shown). Western blot analysis reveals that Akt phosphorylation at serine 473 is
completely absent in the *Rictor*-null macrophages as expected and phosphorylation of FOXO1 is also reduced. STAT6 phosphorylation is also normal in the *Rictor*-null BMDMs, unlike the *Rictor*-null T-cells. Interestingly, unlike the *Mac-Tsc1KO* BMDMs, phosphorylation of Akt at threonine 308 is intact in *Rictor*-null BMDMs, although there is a downward mobility shift likely due to decreased serine 473 phosphorylation (Figure 3b). One study suggests that Akt phosphorylation at threonine 308 is most critical for activity, whereas the serine 473 site is necessary for FOXO1 phosphorylation and hyperactivation of Akt. Thus, loss of mTORC2 in *Rictor*-null macrophages is not sufficient to produce a defect in M2 polarization, which implicates phosphorylation of Akt at threonine 308 downstream of IRS2/PI3K/PDK1 as a critical event for induction of the M2 genetic program.

**Akt Regulates a Subset of M2 Macrophage Markers**

*Mac-Tsc1KO* BMDMs display reduced mTORC2 activity and attenuated Akt signaling, yet loss of mTORC2 and serine 473 phosphorylation of Akt is not sufficient to produce a defect in M2 polarization. Akt signaling is known to increase with IL-4 stimulation in macrophages, yet the downstream consequences are unclear. To investigate the role of Akt in M2 polarization, we treated WT BMDMs with Aktviii inhibitor, which inhibits Akt phosphorylation and activity. WT BMDMs pre-treated with Aktviii for 1h prior to IL-4 stimulation for 12h and 24h display reduced mRNA expression of the M2 genes Arg-1, Fizz1, Mgl1, and Pgc-1β, all of which are downregulated in *Mac-Tsc1KO* BMDMs (Figure 4a). Consistent with gene expression results, Arginase-1 activity is completely absent in WT BMDMs treated with IL-4+Aktviii at 12h and 24h (Figure 4a). Gene expression of other M2 macrophage markers, such as *Mrc1*, and *Ym1* is not affected by treatment with Aktviii (Figure 4a). Western blot analysis shows that treatment
with Aktviii prior to IL-4 stimulation abolishes Akt phosphorylation at both serine 473 and threonine 308 as observed in Mac-Tsc1KO BMDMs, in addition to decreasing phosphorylation of FOXO1 and GSK3α/β (Figure 4b). Furthermore, STAT6 phosphorylation is unaffected by Aktviii treatment prior to IL-4 stimulation (Figure 4b). A study performed in adipocytes showed that the Akt target, FOXO1, is able to transrepress rosiglitazone-induced PPARγ activity\(^{33}\). Since attenuated Akt signaling leads to reduced phosphorylation and likely increased nuclear content of FOXO1, this could account for the defective M2 polarization observed in Mac-Tsc1KO BMDMs given the importance of PPARγ in this process (Figure 3a). While PPARγ mRNA levels are normal in Mac-Tsc1KO BMDMs (Figure 2c), the transcriptional activity could be disrupted by FOXO1 transrepression. To address this as a potential mechanism, we used the PPARγ agonist troglitazone in conjunction with IL-4 stimulation. Treatment of Mac-Tsc1KO BMDMs with troglitazone and IL-4 for 24h failed to rescue the defective mRNA expression of Arginase-1, Fizz1, Mgl1, and PGC-1β (Figure 4c). Moreover, expression of known PPARγ-dependent genes F fabp 4 and C d 36, are normal in Mac-Tsc1KO BMDMs, and IL-4 synergizes with troglitazone in increasing expression of these genes above IL-4 treatment alone in both Mac-Tsc1KO BMDMs and Tsc1\(^{fl/fl}\) controls (Figure 4c). In conclusion, results from WT macrophages treated with Aktviii inhibitor provides support for attenuated Akt signaling, particularly reduced PDK1 phosphorylation of threonine 308, as a mechanism for defective M2 polarization in Mac-Tsc1KO BMDMs. In addition, FOXO1 transrepression of PPARγ that could occur downstream of attenuated Akt is unlikely the cause for the defective M2 polarization Mac-Tsc1KO BMDMs. Taken together, these results provide evidence that Akt signaling is necessary for induction of M2 macrophage markers.
Figure 1. Constitutive mTORC1 in BMDMs leads to a pro-inflammatory phenotype.

a. Western blot analysis of Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs in the presence or absence of 20nM rapamycin for 15h.
Figure 1 (continued). Constitutive mTORC1 in BMDMs leads to a pro-inflammatory phenotype. b. TNF-α and Interleukin-10 ELISAs in supernatants from Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs stimulated with 10ng/ml LPS for 2h and 6h. c. TNF-α ELISA in supernatants from Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs stimulated for 6h in the presence or absence rapamycin (20nM, 1h pre-treatment).
Figure 2. Defective M2 Polarization in Mac-Tsc1KO BMDMs. a. IL-4 fails to induce M2 genes expression in Mac-Tsc1KO BMDMs. Data plotted as normalized fold expression of Arg1, Fizz1,
and Mgl1 mRNA levels in Tsc1^{fl/fl} and Mac-Tsc1KO BMDMs stimulated with 10ng/ml IL-4 for 24-48h. Arginase activity with 10ng/ml IL-4 for 24-48h as measured by urea production (µg) and normalized to total protein (µg).

Figure 2. Defective M2 Polarization in Mac-Tsc1KO BMDMs. b. PPAR Expression is unaffected in Mac-Tsc1KO BMDMs. Normalized fold expression of Pparδ and Pparγ in Tsc1^{fl/fl} and Mac-Tsc1KO BMDMs stimulated with 10ng/ml IL-4 for 24h.
C.

Figure 2 (continued). Defective M2 Polarization in *Mac-Tsc1KO* BMDMs.
c. Defective *Pgc-1β* mRNA expression is concomitant with reduced palmitic acid oxidation and mitochondrial biogenesis in *Mac-Tsc1KO* BMDMs stimulated with 10ng/ml IL-4 for 24-48h. FA oxidation plotted as fold induction over basal state (n=2). Mitochondrial biogenesis shown as measured by flow cytometry using MitroTracker® Green.

### d.

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**Figure 2 (continued):** Defective M2 Polarization in *Mac-Tsc1KO* BMDMs. d. STAT6 phosphorylation and nuclear translocation is intact in *Mac-Tsc1KO* BMDMs. Immunoblot analysis of p-STAT<sup>Tyr641</sup> and Total Stat6 in *Tsc1<sup>fl/fl</sup>* and *Mac-Tsc1KO* BMDMs stimulated with 10ng/ml IL-4 for 15min-2h. Nuclear/cytosolic fractions from *Tsc1<sup>fl/fl</sup>* and *Mac-Tsc1KO* BMDMs stimulated with 10ng/ml IL-4 for 30min-1h were blotted with p-STAT<sup>Tyr641</sup> as well as LaminA/C and α-Tubulin to verify fractionation.
**e.**

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**p-STAT6^{Tyr641}**

**α-Tubulin**

**Lamin A/C**

![Bar charts showing nuclear and cytosolic p-STAT6/α-Tubulin levels](chart.png)
Figure 2 (continued): Defective M2 Polarization in Mac-Tsc1KO BMDMs. e. Nuclear/cytosolic fractions from Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs stimulated with 10ng/ml IL-4 for 30min-1h were blotted with p-STAT6<sup>Tyr641</sup> as well as LaminA/C and α-Tubulin to verify fractionation.

Figure 3: Mac-Tsc1KO BMDMs have attenuated IRS2/PI3K/Akt Signaling and Reduced mTORC2 Activity. a. Immunoblot analysis of Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs stimulated with 10ng/ml IL-4 for 15min-2h to examine IRS2/PI3K/Akt Signaling. Mac-Tsc1KO BMDMs have reduced IL-4-induced Akt phosphorylation and well reduced mTORC2 activation.
**b.**

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**Figure 3 (continued).** *Mac-Tsc1KO* BMDMs have attenuated IRS2/PI3K/Akt Signaling and Reduced mTORC2 Activity. **b.** Immunoblot analysis of *Rictor<sup>fl/fl</sup>* and *Rictor<sup>fl/fl</sup>Ub-Cre* BMDMs stimulated with 10ng/ml for 5min-2h. *Rictor<sup>fl/fl</sup>Ub-Cre* BMDMs have reduced Akt<sup>S473</sup> phosphorylation, but intact Akt<sup>Thr308</sup> phosphorylation with IL-4 stimulation.
Figure 3 (continued). Mac-Tsc1KO BMDMs have attenuated IRS2/PI3K/Akt Signaling and Reduced mTORC2 Activity  

**c.** M2 polarization in Rictor<sup>fl/fl</sup>Ub-Cre BMDMs is not defective. Arginase activity with 10ng/ml IL-4 for 12-48h as measured by urea production (µg) and normalized to total protein (µg). Normalized fold expression of Arg-1 and Pgc-1β mRNA levels are shown in Rictor<sup>fl/fl</sup> and Rictor<sup>fl/fl</sup>Ub-Cre BMDMs stimulated with 10ng/ml for 12-48h.
Figure 4. Akt Regulates a Subset of M2 Genes Independent of FOXO1. a. Pharmacological inhibition of Akt reduces M2 gene expression. Normalized fold expression of Arg1, Fizz1, Pgc-1β, Mgl1, Ym1, and Mrc1 mRNA levels in WT BMDMs treated with 10ng/ml IL-4 for 12h or 24h in the presence or absence of 10µM Aktviii, 1h pre-treatment.
Figure 4 (continued). Akt Regulates a Subset of M2 Genes Independent of FOXO1.
b. Immunoblot analysis of WT BMDMs stimulated with 10ng/ml for 30min-2h in the presence of absence of 10µM Aktviii, 1h pre-treatment.
Figure 4 (continued). Akt Regulates a Subset of M2 Genes Independent of FOXO1. c. Transrepression of PPARγ by FOXO1 does not account for defective M2 polarization in Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs. Tsc1<sup>fl/fl</sup> and Mac-Tsc1KO BMDMs treated with 10ng/ml IL-4 in the presence or absence of 1μM troglitazone (co-treatment) for 24h. Normalized fold expression of Arg1, Fizz1, Pgc-1β, Mgl1, Fabp4, and Cd36 mRNA levels are shown.
Discussion

In this study, we use a novel model of myeloid-specific Tsc1 deletion and constitutive mTORC1 activity to elucidate mTOR function in macrophages. Consistent with previous findings in Pan et al 2012, we show that Tsc1 deficiency in macrophages leads to a pro-inflammatory phenotype upon stimulation with LPS, with Mac-Tsc1KO BMDMs displaying increased secretion of pro-inflammatory TNF-α and decreased secretion of anti-inflammatory IL-10 relative to Tsc1<sup>fl/fl</sup> controls. We show, for the first time, that IL-4 stimulated Mac-Tsc1KO BMDMs have defective M2 polarization displaying reduced mRNA expression of key M2 markers Arginase-1, Fizz1, Mgl1/2 and the PPARγ transcriptional coactivator, Pgc-1β relative Tsc1<sup>fl/fl</sup> controls. Mac-Tsc1KO BMDMs also exhibit defects in IL-4 induced mitochondrial biogenesis and fatty acid oxidation, PGC-1β-dependent processes. Examination of IL-4 signal transduction reveals that Mac-Tsc1KO BMDMs have reduced IRS2 stability, attenuated Akt signaling, and diminished mTORC2 activity due to the mTORC1 negative feedback loops that inhibit upstream IRS/PI3K signaling. Loss of mTORC2 and mTORC2-mediated phosphorylation of Akt at serine 473 that occurs in Rictor-null macrophages is insufficient to produce a defect in M2 polarization. Instead, intact Akt threonine 308 phosphorylation in response to IL-4 may critical downstream of IL-4R signaling for induction of M2 genes. Consistent with this, pharmacological inhibition of Akt with Aktviii inhibitor produces a defect in M2 polarization and blocks phosphorylation of Akt at both, serine 473 and threonine 308, mimicking the effects of the mTORC1-mediated negative feedback loops in the Mac-Tsc1KO BMDMs. The data thus support a critical role for IRS2/PI3K activation of PDK1 and phosphorylation of Akt at threonine 308 downstream of the IL-4 Receptor in parallel
with the JAK/STAT6 signal transduction pathway for maximal induction of the M2 polarization program in macrophages.

Several published reports highlight the importance of Akt in macrophage polarization. A recent study proposes that different Akt isoforms regulate M1 versus M2 polarization in macrophages, with Akt1 being important for M2 polarization and Akt2 being important for M1 polarization through differential regulation of miR155 and the transcription factor C/EBP-β. The investigators, however, use LPS stimulation to examine expression M2 gene markers instead of the Th2 cytokine IL-4. Furthermore, expression of Akt2 is controversial in macrophages, as one study demonstrates that Akt1 is the predominant isoform in both bone marrow derived macrophages and peritoneal macrophages, while levels of Akt2 are undetectable. Another recent study implicates Akt in the regulation of M2 macrophage proliferation and enhancement both in vitro and in vivo. In this study, investigators use IL-4Rα−/− mice that have defective engagement of IRS2/PI3K/Akt in response to IL-4 to show that Akt signaling is necessary for M2 macrophage polarization during helminth infection, a Th2-driven process. Our study elaborates on these findings by examining Akt signaling downstream of IL-4R in M2 polarization, as well as defining a specific subset of genes whose expression is downregulated when IRS2/PI3K/Akt signaling is disrupted, either by mTORC-1 mediated negative feedback loops or by pharmacological inhibition of Akt. Genes such as Arginase-1, Fizz1, the C-type lectins Mgl1/2 and Pgc-1β have all been implicated as STAT6-dependent genes through analysis STAT6−/− macrophages. Our data shows that STAT6 signaling is intact in both the Mac-Tsc1KO BMDMs and with pharmacological inhibition of Akt, which demonstrates that IRS2/PI3K/Akt signaling is critical for induction of key M2 markers independent of STAT6. A surprising finding of our
study was the lack of PPARγ and PPARδ involvement in the phenotype of the Mac-Tsc1KO BMDMs, given their established importance in M2 polarization. mTORC1 was shown to have differing effects on PPAR isoforms in various tissues. For example, in Tsc1KO liver, mTORC1 inhibits PPARα-mediated beta oxidation and ketogenesis, whereas in Tsc1KO adipocytes, mTORC1 drives PPARγ-mediated adipogenesis\(^7,37\). The inability of the PPARγ agonist troglitazone to rescue the M2 polarization defect in our model rules out the possibility of direct mTORC1 regulation of PPARγ. While we cannot definitively rule out mTORC1 inhibition of PPARδ, decreased Pgc-1β in Mac-Tsc1KO BMDMs is likely more significant for the observed phenotype in these cells. A previous report shows that PGC-1β is necessary for induction of Arginase-1 activity and the metabolic program to support energy demands of M2 macrophages through the regulation of fatty acid oxidation\(^22\). The current model proposes that IL-4 signaling leads to JAK/STAT6 activation and STAT6 subsequently increases transcription of M2 genes, including PGC-1β\(^14\). PGC-1β then serves as a transcriptional coactivator of STAT6 to increase genes involved in oxidative metabolism as well as to increase expression and activity of PPARγ and –δ\(^14\). Our findings in Mac-Tsc1KO BMDMs implicate PI3K/Akt signaling downstream of IL-4R as a necessary event to increase PGC-1β expression. It is plausible that the parallel IRS2/PI3K/Akt signaling pathway is necessary for upregulation of Pgc-1β expression, which then coactivates STAT6 transcription of the M2 genes Arg1, Fizz1, and Mgl1/2; however, other approaches to assess STAT6 DNA binding and transcriptional activity would be necessary to confirm this in Mac-Tsc1KO BMDMs.

A key experiment to demonstrate that the negative feedback inhibition by mTORC1 upstream leads to the M2 polarization defect would be to pharmacologically
inhibit mTORC1 with the allosteric inhibitor, rapamycin. In Tsc1-deficient hepatocytes and MEFs, rapamycin treatment relieves the negative feedback loops and leads to increased Akt phosphorylation at both serine 473 and threonine 308\(^2,6\). While rapamycin is a specific inhibitor of mTORC1, at certain concentrations and time points, this drug has off target effects, most notably inhibition of mTORC2\(^38\). Given that M2 polarization experiments require a long incubation with IL-4 for Arginase-1 activity and for metabolic assays, it would be difficult to draw conclusions with the possibility of both known and unknown off target effects. Knockdown of raptor, the key component of mTORC1, would be more suitable to address this question, but these studies are challenging in primary macrophages.

The PI3K/Akt/mTOR pathway is a key nutrient and energy sensing pathway activated in response to insulin and other growth factors\(^5,38\). Chronic activation of mTORC1 by nutrient excess, as observed in obesity, leads to inhibition of the insulin response through negative feedback loops, which has pathological consequences for tissues, such as adipose, muscle, and liver\(^1,5\). The combination of increased glucose output by liver, increased insulin production by pancreatic beta cells, and the increase in circulating fatty acids due to unrestrained adipose tissue lipolysis observed in obesity creates a milieu that bombards peripheral tissues with nutrients leading to aberrant mTORC1 activity in many tissues\(^1\). The negative feedback inhibition of mTORC1 on upstream PI3K/Akt signaling that likely serves to maintain inducibility of these signals, becomes chronically inhibited leading to attenuation of insulin responses and ultimately, insulin resistance that precedes Type 2 Diabetes development\(^6\). A chronic low-grade inflammation also accompanies metabolic dysfunction in key insulin responsive tissues in obesity, such as liver and adipose tissue, where resident macrophages undergo a
phenotypic switch from anti-inflammatory M2 macrophages to pro-inflammatory M1 macrophages, further exacerbating insulin resistance\textsuperscript{16}. We believe that *Mac-Tsc1KO* macrophages could serve as model for adipose tissue macrophages during obesity. Macrophages in adipose tissue are bathed with the same excess nutrients that other peripheral tissues encounter, and we believe this could lead to chronic mTORC1 activation. The elevated mTORC1 activity then inhibits upstream IRS2/PI3K/Akt signaling rendering the macrophages “resistant” to IL-4, and thus unable to polarize to an M2 phenotype. Macrophages with elevated mTORC1 also display enhanced pro-inflammatory responses to TLR4 ligand LPS, a response that could be elicited by increased saturated fatty acid binding to TLR4 in obesity\textsuperscript{17}. In the future, it would be interesting to examine mTORC1 and IL-4 signaling in macrophages from mice with a genetic model of obesity, such as *ob/ob* mice, or from mice fed a high fat diet. It would also be interesting to see if mice with *Tsc1* deletion in myeloid lineage cells would have worsened obesity and metabolic dysregulation on a high fat diet. Unfortunately, due to epileptic seizures and premature death in *Mac-Tsc1KO* mice, these studies are not feasible. Perhaps utilization of another genetic model could better address the macrophage mTOR signaling in obesity.

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Ruckerl, D. et al. Induction of IL-4Ralpha-dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo. *Blood* 120, 2307-2316, doi:10.1182/blood-2012-02-408252 (2012).


CURRICULUM VITAE

VANESSA A. BYLES

23 First Street
Groton, CT 06340
(860)-912-9200
Born: 1986
vbyles@gmail.com

Education

Boston University School of Medicine, Boston, MA
January 2011-Present
Master of Arts in Nutrition and Metabolism.
Thesis: The role of Mammalian Target of Rapamycin (mTOR) in macrophage polarization.
Expected Completion Date: January 2013
Cumulative GPA: 3.96

Boston University, Boston, MA
January 2005- May 2008
Bachelor of Science in Human Physiology, Cum Laude, May 2008.
Relevant Courses: Introductory Biology, General Chemistry, Organic Chemistry, Biochemistry, Human Physiology, Gross Human Anatomy, Neuroanatomy/Physiology, Calculus 1 and 2.
Activities: Boston University Admissions Ambassador, Treasurer for Global Alliance for the Immunization of AIDS, Teaching Assistant for Physiological Psychology, and Sigma Alpha Lambda Honors Society member.
Awards: Dean’s List
Cumulative GPA: 3.47

Publications

1. Vanessa Byles¹, Lijia Zhu¹, Jenna D. Lovaas, Laura K. Chmilewski, Joyce Wang, Douglas V. Faller and Yan Dai. “SIRT1 Induces EMT by Cooperating with EMT Inducing Transcription Factors and Enhances Prostate Cancer Migration and Metastasis.” Oncogene. 2012; 31: 4619-29. ¹Authors contributed equally

2. Tomohiko Murakami¹, Johan Ockinger¹, JiuJiu Yu², Vanessa Byles², Aisleen McColl, Aldebaran Hofer, and Tiffany Horng. “Critical Role for Calcium Mobilization in Activation of the Nlrp3 Inflammasome.” PNAS. 2012; 109: 11282-7. ¹Authors Contributed Equally, ²Authors Contributed Equally

and Tumor Cell Invasion in Prostate Cancer Cells." *Prostate.* 2012.


**Research Experience**

Harvard School of Public Health, Department of Genetics and Complex Diseases

November 2008-Present

I am currently working as a research assistant while I complete my Master’s thesis investigating the role of mTOR kinase in macrophage polarization. Specifically, I am focused on how mTOR Complex 1 influences the inflammatory phenotype and metabolism of macrophages, which is relevant in the pathological context of obesity.

**Posters, and Talks:**

2. Anthony Covarrubias¹, **Vanessa Byles¹**, and Tiffany Horng. “The Role of mTOR in the Innate Immune System.” Poster presented at the HSPH Immunology and Infectious Disease Department Retreat, April 2012 at the Harvard Club, Boston. ¹Contributed Equally.

**Teaching Experiences:**

1. August 2010- June 2011. Trained a Master’s student from Uppsala University, Sweden who completed a Master’s thesis studying the link between defective autophagy and inflammation in macrophages.
2. August 2011- January 2012. Trained a second Master’s student from Uppsala University, Sweden who completed a Master’s thesis project characterizing the phenotype of Raptor-deficient macrophages.
3. January 2012- April 2012. Trained two undergraduates from Boston University as laboratory technicians.
4. June-August 2012- Biological Sciences in Public Health Summer Program mentor at Harvard School of Public Health.
June 2007-January 2009
I worked as a research assistant as an undergraduate investigating the role of SIRT1, an NAD-dependent histone deacetylase, in human prostate cancer progression and metastasis. The projects I worked on have culminated in three manuscript publications. My responsibilities included design and completion of experiments as well as data organization and presentation.

**Summary of Research Skills**

I have experience in prostate cancer research using human cancer cell lines and tissue samples. My most recent experience is in areas of macrophage biology, including Toll-like receptor signaling, inflammasome activation, ER stress pathways, autophagy, and M1/M2 macrophage polarization. I am proficient in cell culture, isolating macrophages from bone marrow, gel electrophoresis (DNA and protein), Western blotting, immunoprecipitation techniques, immunostaining, immunohistochemistry, fluorescent microscopy, RNA extraction, quantitative PCR, ELISA, cloning techniques, and retroviral infection of primary macrophages and cell lines. I also have extensive experience with Fluorescence Activated Cell Sorting (FACS), in vitro cell migration and invasion assays, macrophage phagocytosis assays, and metabolic assays, such as de novo lipogenesis and fatty acid oxidation using radiolabeled substrates. I also have 3+ years experience working with mice.

**Volunteer Experience**

**Horizons for Homeless Children**
Cambridge, MA
August 2009- September 2011
Playspace Activity Leader at Hildebrand Family Self-Help Shelter.

**Work Experience**

**Mystic Seaport**
Mystic, CT
July 2005-August 2006
I worked as a cashier with occasional catering jobs. My responsibilities included customer service, food preparation, and restaurant maintenance.

**Hollister, Co.**
Waterford, CT
October 2004-January 2006
I worked as a brand representative and cashier. My responsibilities included assisting customers in the store, updating floor sets weekly, and organizing the stockroom.

**Go Fish Restaurant**
Mystic, CT
September 2004-December 2004
I worked as a hostess at an upscale seafood and sushi restaurant. My responsibilities included customer service, taking reservations, seating customers, and maintaining the host station.