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Myeloid-Specific Rictor Deletion Induces M1 Macrophage Polarization and Potentiates In Vivo Pro-Inflammatory Response to Lipopolysaccharide

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Abstract

The phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) axis plays a central role in attenuating inflammation upon macrophage stimulation with toll-like receptor (TLR) ligands. The mechanistic target of rapamycin complex 2 (mTORC2) relays signal from PI3K to Akt but its role in modulating inflammation in vivo has never been investigated. To evaluate the role of mTORC2 in the regulation of inflammation in vivo, we have generated a mouse model lacking Rictor, an essential mTORC2 component, in myeloid cells. Primary macrophages isolated from myeloid-specific Rictor null mice exhibited an exaggerated response to TLRs ligands, and expressed high levels of M1 genes and lower levels of M2 markers. To determine whether the loss of Rictor similarly affected inflammation in vivo, mice were either fed a high fat diet, a situation promoting chronic but low-grade inflammation, or were injected with lipopolysaccharide (LPS), which mimics an acute, severe septic inflammatory condition. Although high fat feeding contributed to promote obesity, inflammation, macrophage infiltration in adipose tissue and systemic insulin resistance, we did not observe a significant impact of Rictor loss on these parameters. However, mice lacking Rictor exhibited a higher sensitivity to septic shock when injected with LPS. Altogether, these results indicate that mTORC2 is a key negative regulator of macrophages TLR signalling and that its role in modulating inflammation is particularly important in the context of severe inflammatory challenges. These observations suggest that approaches aimed at modulating mTORC2 activity may represent a possible therapeutic approach for diseases linked to excessive inflammation.

Introduction

The innate immune system plays a fundamental role in protecting organisms against a variety of infectious agents. Macrophages are a heterogeneous group of cells with phagocytic activity that are fulfilling key roles in innate immunity[1]. Macrophages digest and kill pathogens and help maintain tissue homeostasis by removing dead cells and debris. In response to pathogens, these cells produce several pro-inflammatory cytokines and chemokines that contribute to infection clearance. Over the last decades, various macrophage subsets with distinct immune functions have been described[2]. Classically activated macrophages (M1 macrophages) have pro-inflammatory functions and mediate host defense against various pathogens and exert anti-tumor immune responses. Alternatively activated macrophages (M2 macrophages), on the other hand, exhibit anti-inflammatory roles and regulate tissue homeostasis in processes like wound healing.

Toll-like receptors (TLR) are molecular pattern recognition receptors recognizing danger signals derived from a plethora of infectious agents [3]. Macrophages express these receptors and react to such receptor ligation. Indeed, upon ligand binding, TLR molecules recruit signaling adapters and initiate a pro-inflammatory response that culminates with the activation of several transcription factors that turn on the expression of pro-inflammatory genes required for pathogen clearance. Although the importance of TLR signalling in immune defense is well known, an exaggerated inflammatory response can also seriously impair the functions of tissues and lead to septic shock and death. Uncontrolled activation of TLR in macrophages contributes to the development of autoimmune diseases and atherosclerosis and promotes the susceptibility to tumor metastasis[4]. Along with cancer, chronic low-grade inflammation plays a critical role in the development of obesity and insulin resistance[5]. In this context, gaining insights into the regulation of inflammation could provide new therapeutic approaches to treat human pathologies.
Class I phosphoinositide-3-kinase (PI3K) is a class of lipid kinases that plays diverse roles in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking[6,7]. Upon activation, PI3K is recruited to the plasma membrane, where it phosphorylates phosphatidylinositol (4,5)-biphosphate (PIP2) to generate phosphatidylinositol (3,4,5)-triphosphate (PIP3). Increased PIP3 levels promotes the recruitment of Akt (also known as protein kinase B) to the plasma membrane, causing Akt phosphorylation by 3-phosphoinositide-dependent protein kinase 1 (PDK1) on threonine 308 (Thr308) and by the mechanistic target of rapamycin (mTOR) complex 2 (mTORC2) on serine 473 (Ser473)[8]. These phosphorylation events, that are required for a maximal activation of Akt, allow this kinase to regulate processes including metabolism and survival[9]. Several reports show that the PI3K-Akt axis is a key regulator of the cellular response to TLR ligands[10–12]. Activated TLRs physically recruit and activate PI3K[13–17], promoting an elevation in Akt phosphorylation[13,17–21]. Many studies indicate that PI3K-Akt negatively regulates the expression of several pro-inflammatory genes in response to TLR ligands in macrophages in vitro[10–12]. Consistent with these results, macrophages isolated from myeloid-specific Pkhlk1 knockout mice show a reduction in Akt phosphorylation and an exaggerated pro-inflammatory response to LPS[22]. The fact that Akt deficient macrophages display elevated cytokine production and that mice lacking Akt1 have impaired tolerance to LPS stimulation support the idea that this kinase is a critical element regulating TLR signalling downstream of PI3K[23,24].

The mechanistic target of rapamycin is a key component of a signaling network that senses and integrates a variety of environmental cues to regulate organismal growth and homeostasis[25,26]. This serine/threonine protein kinase interacts with several proteins to form two distinct complexes named mTOR complex 1 (mTORC1) and mTORC2. mTORC2 is activated by growth factors such as insulin through yet poorly defined mechanism(s) requiring PI3K. When active, mTORC2 phosphorylates Akt, serum- and glucocorticoid-induced protein kinase 1 (S6K1), and protein kinase C-ζ (PKC-ζ). The role of mTORC2 in modulating TLR signalling in macrophages is still unclear. Since mTORC2 deletion is shown to impair Akt action towards some, but not all of its substrates[27], it is difficult to predict its implication in the regulation of inflammation. An elegant study published recently has demonstrated that the reduction in mTORC2 function exacerbates the pro-inflammatory response to LPS in dendritic cells (DC) in vitro[18]. The same report revealed that mTORC2 modulates inflammation through Akt activation and Forkhead box O1 (FoxO1) nuclear localization. Whether mTORC2 has a similar impact in macrophages and, most importantly, how its function affects the inflammatory response to acute and chronic inflammation in a physiological context remains to be characterized.

In this report, we show in primary macrophages and cell lines in vitro that the activation of TLRs promotes mTORC2 activity. In order to determine the role of mTORC2 in the macrophage regulation of the inflammation in vivo, we have generated a mouse model lacking the mTORC2 component rapamycin-insensitive companion of mTOR (Rictor) in myeloid cells. Macrophages isolated from these mice exhibit an exaggerated response to TLRs ligands, and express high levels of M1 genes and lower levels of M2 markers. Surprisingly, loss of Rictor did not exacerbate macrophage infiltration in adipose tissue, inflammation and insulin resistance when animals were exposed to a high fat diet, a condition known to promote a chronic, but low-grade inflammatory state. However, mice lacking Rictor exhibited a higher sensitivity to septic shock when injected with LPS. Together, these results indicate that mTORC2 is a key regulator of TLR signalling that plays key roles in the down-regulation of the inflammatory response during acute infection.

Materials and Methods

Cell Lines, Antibodies, and Reagents

Reagents were obtained from the following sources: antibodies to phospho-S473 Akt, phospho-T308-Akt, Akt, mTOR, RICTOR, NDRG1, phospho-T346-NDRG1, phospho-PKCβII-T636/641, phospho-Thr180/Tyr182-p38, phospho-S180/S181-IKKα/β, phospho-Thr202/Tyr204-Erk1/2, phospho-Thr183/Tyr185-JNK from Cell Signaling Technology (cat #4696, 2965, 4691, 2983, 2140, 5196, 3317, 9375, 9211, 2681 and 9101 respectively); antibodies to PKCα and IκBα from Santa Cruz Biotechnology (cat #SC-208 and SC-371). Secondary antibodies were all purchased from Santa Cruz Biotechnology. TLR ligands were purchased from Sigma (LPS, L3012), Integrated DNA technology (CpG), Enzo Life Sciences (Malp2, ALX-162-027-C505; PAM3, ALX-163-066-M022; and R848, ALX-420-038-M005). Primary mouse fibroblasts were established from E13.5 embryos of Rictorlox/lox mice crossed with P53−/− mice, as described previously[27]. Raw264.7 macrophages were purchased from ATCC.

Isolation, Differentiation, and Culture of TEM

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Massachusetts Institute of Technology Committee on Animal Care (CAC) (permit 090908012). For thioglycollate-elicited macrophages (TEM) isolation, mice were injected intraperitoneally (ip) with 1.0 mL of a sterile solution of thioglycollate (3%) (BD Bioscience). Four days later, mice were sacrificed and macrophages collected from a peritoneal cavity wash under aseptic conditions with 10 mL of sterile PBS. Next, macrophages were plated in 5% CO2 humidified incubator at 37°C for 1 hour in RPMI 1640 media supplemented with 10% heat inactivated fetal cell serum (FCS), 1% penicillin-streptomycin (Gibco, 15140-122) and 2 mL 1-glutamine (Gibco, 35050-061). Plates were then washed 5 times with warm PBS to remove non-adherent cells. TEM were allowed to sit for 24 hours and then used for experimentation.

Isolation, Differentiation, and Culture of BMDM

For bone marrow derived-macrophage (BMDM) isolation, mice were sacrificed and both tibias and femur were collected. Skin and muscles were removed and the bones were washed in 70% ethanol. Bone marrow was flushed out the bones using a syringe filled with BDMG growth media. The base of this medium is the same that the one used for TEM, but supplemented with 30% of L-Cell conditioned media. To make L-Cell conditioned media, 2.5×10⁵ L929 fibroblasts (ATCC) were plated into 50 ml of RPMI 1640 media containing 10% FCS, penicillin-streptomycin and glutamine as described above in T175 flasks. Cells were grown to confluence (5 days) and media was centrifuged, filtered and stored frozen in aliquots at −80°C until needed. The bone marrow cells isolated from the tibias and femurs were re suspendedit in BDMG growth media and the cells allowed to differentiate for 6 days. Subsequently, media was changed every two days.
Generation of Macrophage-specific Rictor Null Mice

Rictor<sup>Lox/Lox</sup> mice were produced as described before[28]. These animals were backcrossed 4 times to C57BL/6J and were then crossed to Lysozyme<sup>cre<sup>+/−</sup></sup>; Rictor<sup>Lox/Lox</sup> offspring (where WT refers to wild type) as F1 generation[29]. These heterozygous mice were crossed to obtain the Lysozyme<sup>-Rictor</sup><sup>WT</sup> and their wild-type littersmates that are Lysozyme<sup>-Rictor<sup>−/−</sup></sup>. These heterozygous mice were crossed to obtain the Lysozyme<sup>-Rictor<sup>WT</sup></sup> or genotype Lysozyme<sup>-Rictor<sup>−/−</sup></sup>; Rictor<sup>Lox/Lox</sup> and their wild-type littersmates that are Lysozyme<sup>-Rictor<sup>−/−</sup></sup> (referred to here as Lysozyme<sup>-Rictor<sup>WT</sup></sup>). For all mice used, the genotypes were determined by PCR analysis of tail genomic DNA as described previously[29].

Metabolic Studies with LysM-Rictor<sup>WT</sup> or LysM-Rictor<sup>KO</sup> Mice

Mice (6–8 weeks old) were fed a low fat diet (10% kcal fat, Research Diet, D12450B) or high fat diet (60% kcal fat, Research Diet, D12492) for 21 and 23 weeks respectively. The mice were maintained under temperature- and humidity-controlled conditions with a 12-h light/dark cycle and were allowed to eat and drink water ad libitum. At week 18, mice were fasted overnight before a glucose tolerance test (GTT) on the following morning. Briefly, blood was collected from the tail at time 0, 15, 30, 45, 60 and 120 minutes following a ip injection of glucose (1 g/kg). Glucose levels were measured using a Lifescan OneTouch Ultra glucose meter. At week 19, mice were fasted for 6 hours before the insulin tolerance test. Blood was collected from mouse tail 0, 15, 30, 45, 60 and 120 minutes following an ip injection of insulin (0.75U/kg, Eli Lilly) and glucose levels were measured as described for the GTT. Mice were then allowed to rest for few 2 to 4 weeks. On the day of sacrifice, following an overnight fast, the animals were anaesthetized with isoflurane and killed by cervical dislocation at 2 to 4 weeks. On the day of sacrifice, following an overnight fast, the animals were anaesthetized with isoflurane and killed by cervical dislocation. Tissues were collected and frozen for analysis. Plasma metabolites were measured using commercial kits from different companies; insulin (Ultra sensitive Mouse Insulin ELISA kit, Crystal Chem Inc.), triglycerides (Infinity Triglycerides, Thermo Scientific), and cholesterol (Infinity Total Cholesterol, Thermo Scientific).

In vivo Injection of LPS

Lysozyme<sup>-Rictor<sup>WT</sup></sup> or Lysozyme<sup>-Rictor<sup>KO</sup></sup> mice (6–8 months old) were injected ip with a solution of LPS (2.5 mg/kg) (Sigma Aldrich L3012). Body temperature was monitored over the course of the experimentation using a rectal temperature sensor. Blood was collected before and after LPS injection. Preliminary experiments carried in our laboratory showed that mice have a core body temperature dropping down to 33+/−0.2°C or below, following LPS injection, have a poor recovery rate and a high probability of death and this was set as a criteria for an ethical sacrifice; mice reaching this limit were humanely sacrificed by CO<sub>2</sub> asphyxiation.

Protein Lysates Preparation for Western Blotting

After washing with ice-cold PBS, cells were lysed with Triton-X 100 containing lysis buffer (50 mM HEPES [pH 7.4], 2 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 40 mM NaCl, 50 mM NaF, 2 mM sodium orthovanadate, 1% Triton-X 100, and one tablet of EDTA-free protease inhibitors (Roche) per 25 ml). Cells were then rotated at 4°C for 10 minutes before the soluble fraction of cell lysates were isolated by centrifugation (13,000 rpm, 10 min). Protein levels were quantified using Bradford reagents (BioRad) and analyzed by western blotting using ECL (GE healthcare Life Sciences).

RNA Isolation and qPCR Analysis

Total RNA was isolated from cells or tissues using the RNeasy Kit (Qiagen) and reverse-transcription was performed using Superscript III reverse transcriptase (Invitrogen). The resulting cDNA was diluted in DNase-free water (1:15) before quantification by real-time PCR. mRNA transcript levels were measured using SYBR Green PCR master mix (Applied Biosystems) and the Biosystems 7900HT Sequence Detection System v2.3 software. All Data are expressed as the ratio between the expression of target gene to the housekeeping genes 36B4.

Cytokine Measurements

Cytokines were measured from cell culture media and plasma using commercial ELISA kits from R&D according to manufacturer’s instructions.

Statistical Analysis

Results are expressed as means ± SE. When appropriated, Student’s unpaired t tests or factorial ANOVA followed by Newman-Keuls’ multiple range tests were used for multiple comparisons. P<0.05 was taken as the threshold of significance.

Results

Over the years, several groups observed that TLR stimulation increases the activation of the PI3K-Akt axis[13,17–21]. Supporting these findings, we observed that stimulation of Raw264.7 macrophages with the TLR4 agonist LPS rapidly enhances Akt phosphorylation (Figure 1A). Interestingly, LPS not only induced the phosphorylation of Akt on Thr308, a site phosphorylated by PDK1[30], but also induced the phosphorylation of Ser473, a site phosphorylated by mTORC2[31], suggesting the implication of mTORC2 in the cellular response to TLR stimulation. In order to characterize the implication of mTORC2 in the regulation of PI3K-Akt following TLR activation, we stimulated Rictor<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) with LPS. Similarly to what was observed in Raw264.7 macrophages, LPS induced the phosphorylation of Akt on Thr308 and Ser473 residues in wild-type MEFs (Figure 1B). Interestingly, loss of Rictor completely abrogated the phosphorylation of Ser473, while the phosphorylation of Akt on Thr308 was only partially reduced. These results indicate that mTORC2 plays a necessary role in TLR4-mediated activation of Akt.

Observing that mTORC2 regulates elements of the TLR signalling, we next evaluated the implication of mTORC2 in regulating the functionality of macrophages in vitro and in vivo. To do so, we crossed Rictor<sup>Lox/Lox</sup> mice[28] with mice expressing a Cre-recombinase under the control of the Lysozyme M promoter (Lysozyme<sup>cre<sup>−/−</sup></sup> mice)[29]. This strategy allowed the selective deletion of Rictor in myeloid cells (granulocytes and mature macrophages) (Lysozyme<sup>-Rictor<sup>−/−</sup></sup> mice)[29]. Lysozyme<sup>-Rictor<sup>−/−</sup></sup> mice were born at the expected Mendelian ratio and did not show any obvious abnormality. As shown in Figure 2, the presence of Lysozyme<sup>cre<sup>−/−</sup></sup> in Rictor<sup>Lox/Lox</sup> mice resulted in the recombination of Rictor gene and in the loss of the protein in bone marrow-derived macrophages (BMDM) and in thygoccollate-elicited macrophages (TEM) (Figure 2A, 2B and 2C). Confirming the specificity of our approach, we did not observe any change in RICTOR protein levels in the peripheral tissues of Lysozyme<sup>-Rictor<sup>−/−</sup></sup> mice (Figure 2D). As expected, Rictor<sup>−/−</sup> mice were severely impaired in the phosphorylation of Akt on Ser473 in a dose-dependent manner (Figure 2B and 2C). We also observed that the phosphorylation of protein kinase Cα/β (PKCα/β) was significantly reduced in the BMDM and the TEM of Lysozyme<sup>-Rictor<sup>−/−</sup></sup> mice (Figure 2B and 2C). The phosphorylation of
First, we investigated the basal gene expression in BMDM cells previously shown to be altered by TLR activation in these cells. To mTORC2 deletion in macrophages translates into changes in GSK3β activation and the transcription of several pro-inflammatory genes (Figure 6). Importantly, the modulation of the inflammatory response linked to mTORC2 disruption was not linked to any consistent change in signalling through the MAP kinase, a signalling pathway known to play a key role in promoting the nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) activation and the transcription of several pro-inflammatory genes (Figure 6).

Our findings indicate that mTORC2 is activated upon TLR stimulation and that the loss of complex activity is associated with an exacerbated pro-inflammatory response by macrophages in vitro. To test whether myeloid-cell Rictor deletion is associated with changes in macrophage function in vivo, LysM-Rictor KO mice were either rendered obese, a condition characterized by a chronic low-grade inflammation, or injected with LPS, which mimics an acute, severe septic inflammatory condition. Reports published over the last decade have shown that obesity promotes systemic and low-grade inflammation in humans and rodents and that the inflammatory mediators secreted by activated macrophages play a crucial role in the development of insulin resistance.[5,34–38] Elegant studies have shown that M2 macrophages reside into white adipose tissue (WAT) and that conditions leading to obesity promote the accumulation of M1 macrophages into WAT.[34] Because our in vitro results showed that Rictor loss promotes the expression of pro-inflammatory markers while reducing those of anti-inflammatory cytokines, we hypothesized that LysM-Rictor KO mice could be prone to inflammation and may become more insulin resistant when exposed to a high fat diet. In order to test this hypothesis, LysM-Rictor WT or LysM-Rictor KO mice were fed a low or a high fat diet between 21 to 23 weeks. As shown in Figure 7A and 7B, depleting Rictor in myeloid cells did not significantly affect the growth curves.
in response to chow or high fat diet. Consistent with these results, we observed no difference in tissue weight between LysM-Rictor WT or LysM-Rictor KO mice (Figure 7C and 7D). As expected, feeding mice with a high fat diet increased circulating levels of glucose, insulin, and triglycerides (Figure 7E). However, although LysM-Rictor KO fed a chow diet tended to have a lower glycaemia, we observed no significant difference in blood parameters between LysM-Rictor WT and LysM-Rictor KO mice (Figure 7C and 7D). Supporting these results, glucose and insulin tolerance tests (GTT and ITT respectively) did not reveal any major differences between the genotypes, thus indicating that the depletion of mTORC2 function alone does not deeply affect insulin sensitivity in the context of leanness and obesity in vivo (Figure 7F and 7G).

Figure 2. Production of a macrophage-specific Rictor knock-out mouse model. (A) PCR strategy for the genotyping and identification of wild-type and KO mice. On the left panel is presented the PCR reaction showing the presence or the absence of the Lox allele in the Rictor gene. PCR was performed from a piece of tail collected from mice. The right part of the panel shows the efficiency of the recombination of the Rictor allele in BMDM isolated from the Rictor WT, Rictor Lox/WT, Rictor Lox/Lox expressing or not the LysMcre. The presence of the Δ allele confirms the recombination of the Lox sites and the deletion of the targeted exon. (B to D) Confirmation of the specific loss of RICTOR in (B) BMDM and (C) TEM but not in (D) other mouse tissues. BMDM and TEM were isolated from mice and cultured as described in the methods sections. Proteins were extracted from cells and tissues and lysates were analysed by immunoblotting for indicated proteins. In B, C, and D, each line represents one mouse. (E–F) The loss of Rictor does not affect cell morphology and proliferation of (E) BMDM or (F) TEM.

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Figure 3. TLR activation promotes AKT phosphorylation through the activation of mTORC2. BMDM were isolated from LysM-Rictor WT or LysM-Rictor KO mice and were differentiated for 6 days in vitro. Cells were then plated and incubated 24 hours. Cells were wash twice with PBS, incubated 2 hours in RPMI 0.1% FBS and then treated with (A) LPS (250ng/ml), (B) R848 (0.1μM), or (C) CpG (0.5μM) for the indicated times. Proteins were extracted and lysates were analysed by immunoblotting for indicated proteins.

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LysM-\textbf{Rictor}^{WT} mice (Figure 7H). This was also confirmed histologically (not shown). However, none of the pro-inflammatory genes was increased in \textbf{LysM-Rictor}^{KO} mice (Figure 7H). Similar results were obtained when the expression of these genes was tested in the liver (Figure 7I). These results indicate that \textit{Rictor} loss in myeloid cells does not exacerbate the chronic low-grade inflammation and insulin resistance associated to obesity.

In a second experimental setting, we assessed the role of mTORC2 in an acute inflammatory context namely an ip injection of the TLR4 agonist LPS. LPS injection in mice leads
Figure 5. mTORC2 loss exacerbates the pro-inflammatory profile of BMDM in response to several TLR agonists. (A to D) Impact of several TLR agonists on gene expression in macrophages isolated from LysM-\textsuperscript{Rictor\textsuperscript{WT}} or LysM-\textsuperscript{Rictor\textsuperscript{KO}} mice. BMDM were isolated from LysM-\textsuperscript{Rictor\textsuperscript{WT}} or LysM-\textsuperscript{Rictor\textsuperscript{KO}} mice and were differentiated for 6 days \textit{in vitro}. Cells were then plated and incubated 24 hours. Cells were then treated with (A) PAM3 (1ug/ml), (B) MALP2 (0.1ug/ml), (C) R848 (0.1uM), or CpG (0.5uM) for 8 hours. mRNA expression of markers of the classically activated (M1) and alternatively activated (M2) macrophages were measured by qRT-PCR and normalized to 36B4 mRNA levels. Data are expressed as the mean $\pm$ SEM for n = 4 per condition. *$p<0.05$ versus control. This experiment was repeated twice and similar results were observed.

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Interestingly, we observed that the survival rate of mice that did not survive LPS versus the control mice (Figure 8C). The body temperature of mice was significantly reduced over the first 4 hours following LPS injection (Figure 8A). Such a hypothermic response has been observed several times in rodents injected with LPS[39]. Preliminary experiments carried in our laboratory showed that mice having a drop in the core body temperature down to 33+/−0.2°C or below following LPS injection have a poor recovery rate and a high probability of death. In order to limit pain, any mouse reaching this limit was humanely sacrificed.

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The body temperature of mice was significantly reduced over the first 4 hours following LPS injection (Figure 8A). Such a hypothermic response has been observed several times in rodents injected with LPS[39]. Preliminary experiments carried in our laboratory showed that mice having a drop in the core body temperature down to 33+/−0.2°C or below following LPS injection have a poor recovery rate and a high probability of death. In order to limit pain, any mouse reaching this limit was humanely sacrificed.

Interestingly, we observed that the survival rate of LysM-RictorKO mice was reduced compared to control animals (Figure 8B). Indeed, 64% of LysM-RictorKO mice were sacrificed within 24 hours following LPS injection, compared to only 17% of LysM-RictorWT mice. Importantly, we noted a significant increase in the circulating levels of TNFα in the plasma of LysM-RictorKO mice that did not survive LPS versus the control mice (Figure 8C). The overproduction of TNFα is known as an important factor contributing to hypothermia in response to LPS injection in rodents[40]. These results indicate that, similarly to what observed in vitro, the depletion of mTORC2 in macrophages exacerbates inflammation in response to TLR activation in vivo.

Discussion

Although the importance of TLR signalling in the host response to infections has been well demonstrated, an exaggerated inflammatory response can seriously impair the functions of tissues and lead to septic shock and death. Additionally, unrestrained activation of TLR can contribute to the development of autoimmune diseases and atherosclerosis and promote the susceptibility to tumor metastasis[4]. These observations indicate that the benefits linked to TLR signalling activation follow a U-shaped curve, where too little activity reduces the ability of the host to fight infection, whereas too much impairs tissue function and systemic homeostasis. The results presented in this report support previous literature showing that the PI3K signalling plays a fundamental role in dampening inflammation in response to TLR activation. Importantly, we show that mTORC2 is required for the maximal activation of Akt in response to TLR ligands and that defects in mTORC2 promote the skewing of macrophages towards the M1 phenotype and exacerbates the inflammatory response in vivo.

mTORC2 Regulates TLR Signaling

Stimulation of myeloid cells with TLR ligands promotes PI3K signalling[13,17–21]. Although several mechanisms linking TLR signalling to PI3K have been proposed[13–17], recent reports indicate that B-cell adapter for PI3K (BCAP) plays a key role in...
Figure 7. Myeloid-specific deletion of Rictor does not affect metabolic homeostasis in mice fed a low or a high-fat diet. (A–D) Body and tissue weight of LysM-RictorWT or LysM-RictorKO mice fed a (A and C) low or (B and D) high fat diet for 21 and 23 weeks respectively. (E) Blood parameters of LysM-RictorWT or LysM-RictorKO mice fed either a low or a high fat diet. Mice were fasted for 6 hours before blood collection. ▽ denotes a significant difference between low fat fed and high fat fed mice (p < 0.05). The p value for the genotype effect is indicated on the right side of the table. (F–G) GTT and ITT of LysM-RictorWT or LysM-RictorKO mice fed with (F) low or (G) high fat diet. For the panels A to G, n = 6–13 mice per group. (H–I) Gene expression in (H) WAT and (I) liver of LysM-RictorWT or LysM-RictorKO mice fed a low or a high fat diet. mRNA expression was measured by qRT-PCR and normalized to 36B4 mRNA levels. Data are expressed as the mean ± SEM for n = 6–10 per condition. ▽ denotes a significant difference between low fat fed and high fat fed mice (p < 0.05). * denotes a significant difference between LysM-RictorWT or LysM-RictorKO mice (p < 0.05). doi:10.1371/journal.pone.0095432.g007
this process[13,17]. BCAP exhibits a constitutive phosphorylation state on tyrosine residues and has the ability to bind the p85 subunit of PI3K [17]. Upon LPS stimulation, a pool of BCAP associated to PI3K is transiently recruited to the plasma membrane, which facilitates PI3K production by PI3K[17]. From a functional perspective, loss of BCAP severely impairs the activation of PI3K-Akt in response to TLR stimulation and that BCAP deficient mice show an exaggerated pro-inflammatory response to TLR stimulation and bacterial infection[13,17]. Interestingly, these observations are very similar to what we observed here following the deletion of Rictor, an essential component of mTORC2. Because mTORC2 activation depends on PI3K, this strongly suggests that mTORC2 is one important mediator of the effect of PI3K on TLR signalling.

mTORC2 is activated by PI3K and, together with PDK1, regulates the activity of Akt. By directly phosphorylating Akt on Ser473 and Thr308, mTORC2 and PDK1 insure the maximal activation of this kinase[30,31]. The functional relevance of Akt in the modulation of TLR stimulation was demonstrated by the findings that direct inhibition of Akt in monocytes promotes a pro-inflammatory phenotype similar to that observed following PI3K inhibition[41]. Supporting these results, Akt deficient macrophages show elevated cytokine production and mice lacking Akt1 have impaired tolerance to LPS stimulation[23,24]. It was demonstrated that Akt modulates TLR activation through several mechanisms. Akt phosphorylates and inactivates GSK3β, which reduces NF-KB activation and promotes the expression of the anti-inflammatory cytokine IL-10[41]. Administration of a GSK3β inhibitor, which mimics PI3K-Akt activation, potently suppressed the pro-inflammatory response in mice receiving LPS. Akt has also been shown to reduce inflammation through the transcription factor FoxO1. When active, Akt phosphorylates FoxO1, which promotes its nuclear exclusion[42]. Knockdown or deletion of FoxO1 in various cell type was shown to reduce the expression of pro-inflammatory mediators and to promote the expression of anti-inflammatory cytokines[18,43–46].

Confirming previous findings[13,17–21], we observed that stimulation of myeloid cells with TLR agonists promotes Akt phosphorylation on the sites regulated by both mTORC2 and PDK1. Importantly, we showed that Rictor deletion completely blocks TLR ligand-mediated Akt phosphorylation on Ser473, while Akt phosphorylation on the Thr308 residue was only partially impaired. Rictor loss was also associated with a reduction in the activity of SGK1, as illustrated by the reduction in NDRG1 phosphorylation. We observed that the reduction in mTORC2 activity induced by Rictor deletion was linked to a reduction in the phosphorylation of FoxO1/3, but not of GSK3β. This observation is particularly interesting considering that FoxO1/3 plays a crucial role in regulating inflammation in response to TLR ligands[43]. Here, we observed that the loss of the essential mTORC2 component Rictor in macrophages strongly promoted the expression of pro-inflammatory M1 genes in response to TLR ligands. We also noted a consistent reduction in the expression of several M2 markers in Rictor depleted cells. As shown in macrophages lacking BCAP, the pro-inflammatory phenotype linked to Rictor deletion was not associated with any consistent modulation of MAP kinase and NF-KB signalling[17]. Interestingly, one recent report revealed that Rictor loss in DCs promotes the expression of pro-inflammatory markers in response to TLR4 stimulation, an effect that does not depend on MAP kinases and NF-KB[16]. Brown et al. showed that overexpression of a constitutively active Akt blocks the pro-inflammatory phenotype linked to Rictor deletion and that the mTORC2-Akt axis regulates inflammation by controlling FoxO1 nuclear localization[18]. Altogether, these observations indicate that mTORC2 is required for the regulation of FoxO1/3 in response to TLR signalling and that this kinase serves as a key negative regulator of inflammation in DCs and macrophages in vivo. Our observations also demonstrate a key role of mTORC2 in regulating macrophage polarization.

One important aim of our work was to determine the in vivo impact of mTORC2 deletion in macrophages. Our first approach was to induce the development of obesity in control or LysM-RictorKO mice by exposing them to a high fat diet. Obesity is recognized as a low grade but chronic inflammatory disease[39,47–49]. Weight gain promotes macrophage infiltration in WAT, their M1 polarization, and the expression of several pro-inflammatory cytokines[34–38,50]. Free fatty acids, whose concentration is high in obesity, have been shown to activate TLR4 and to promote cytokine production and inflammation[51,52]. The elevation in cytokine levels causes systemic insulin resistance by reducing the efficiency of the insulin signalling cascade in peripheral tissues[53]. Because LysM-RictorKO macrophages showed an exaggerated response to TLR stimulation in vivo, we...
hypothesized that LysM–Rictor KO mice would be prone to inflammation and insulin resistance when chronically fed a high fat diet. Although high fat feeding contributed to promote inflammation, macrophage infiltration in WAT and systemic inflammation, we did not observe a significant impact of inflammation, macrophage infiltration in WAT and systemic fat diet. Although high fat feeding contributed to promote inflammation and insulin resistance when chronically fed a high fat diet increased macrophages migration to WAT, M1 macrophages polarization, the expression of pro-inflammatory genes in WAT, and insulin resistance[46]. Strikingly, all the effects linked to Ptdk1 loss were corrected by overexpressing a dominant negative form of FoxO1 in macrophages, indicating that PDK1 affects inflammation essentially through its effect on the Akt-FoxO1 axis. The results presented by Kawano et al. greatly differ from ours. It is possible that the discrepancy between our studies could relate to a different residual Akt activity remaining in myeloid cells following Rictor or PDK1 deletion. Here, we observed that the loss of Rictor minimally affected the basal phosphorylation of Akt on Thr308, the site regulated by PDK1. In response to TLR ligands, LysM–Rictor KO macrophages showed only a partial defect in Thr308 phosphorylation. Importantly, studies in mTORC2-deficient MEFs have shown that maintenance of Thr308 phosphorylation alone empowers Akt with enough activity to phosphorylate many of its substrates[27,33,54]. The fact that PDK1 loss completely abrogates Thr308 phosphorylation could explain the differences observed between our reports. From a different perspective, we cannot exclude the possibility that differences in the genetic background of mice or variation in the composition of the diet could have altered the severity of the inflammatory responses and the outcome of our studies.

To determine the importance of mTORC2 for the regulation of the inflammatory response in the context of severe and acute infection, we have tested the impact of LPS injection in LysM–Rictor KO mice. We observed that deletion of Rictor in myeloid cells have exacerbated systemic inflammation following a LPS challenge. The levels of circulating TNFα were significantly higher in mice lacking the mTORC2 component Rictor. Moreover, LysM–Rictor KO mice showed a more pronounced reduction in body temperature following the LPS challenge. Such hyper responsiveness to LPS was observed in mice lacking PI3K[53], BCAP[13,17], PDK1[22], or Akt1[23,24] and in mice treated with PI3K inhibitors[56]. Conversely, a reduction in inflammation was reported in mice lacking phosphatase and tensin homolog (PTEN), a negative regulator of PI3K signalling[55]. Interestingly, several reports showed that insulin, a very potent activator of PI3K, significantly reduces the severity of endotoxemia in rodents injected with LPS[57,58]. When combined, these observations clearly show that PI3K signalling plays a crucial role in reducing innate inflammation in response to TLR stimulation in vivo. Our findings support this model and further confirm that mTORC2, downstream of PI3K, is involved in this process.

In vivo experiments with LysM–Rictor KO mice revealed that the loss of mTORC2 minimally affected the severity of inflammatory response in obese animals, but had a significant impact in mice exposed to LPS. This indicates that the anti-inflammatory properties of mTORC2 depend on the severity of the inflammatory challenge. These interesting observations support the idea that activation of PI3K signalling by TLR stimulation has evolved to protect the host against the negative impact linked to excessive inflammation. Obesity, which is linked to a low grade but chronic inflammatory state, may not represent a sufficient inflammatory challenge requiring the retro-inhibition of TLR by PI3K signalling. Overall, our results indicate that mTORC2 is a key negative regulator of macrophages TLR signalling and support the idea that this protein complex could be targeted to modulate inflammation, especially in the context of severe inflammatory challenges.

Author Contributions

Conceived and designed the experiments: WFF PP ML. Performed the experiments: WFF PP ML. Analyzed the data: WFF PP ML. Contributed reagents/materials/analysis tools: DMS. Wrote the paper: WFF PP DMS ML.

References


