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mTORC2 Deficiency in Myeloid Dendritic Cells Enhances Their Allogeneic Th1 and Th17 Stimulatory Ability after TLR4 Ligation In Vitro and In Vivo

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The mammalian/mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine-threonine kinase. It regulates cell growth, metabolism and proliferation (1), and immune cell function (2, 3) in response to environmental cues (4). mTOR performs its catalytic function in at least two independent complexes: mTOR complex (mTORC)1 and mTORC2. In contrast to the well-defined role of mTORC1 in dendritic cells (DC), little is known about the function of mTORC2. In this study, to our knowledge, we demonstrate for the first time an enhanced ability of mTORC2-deficient myeloid DC to stimulate and polarize allogeneic T cells. We show that activated bone marrow–derived DC from conditional Rictor−/− mice exhibit lower co-inhibitory B7-H1 molecule expression independently of the stimulus and enhanced IL-6, TNF-α, IL-12p70, and IL-23 production following TLR4 ligation. Accordingly, TLR4-activated Rictor−/− DC display augmented allogeneic T cell stimulatory ability, expanding IFN-γ+ and IL-17+, but not IL-10+ or CD4+Foxp3+ regulatory T cells in vitro. A similar DC profile was obtained by stimulating Dectin-1 (C-type lectin family member) on Rictor−/− DC. Using novel CD11c-specific Rictor−/− mice, we confirm the alloreactive Th1 and Th17 cell-polarizing ability of endogenous mTORC2-deficient DC after TLR4 ligation in vivo. Furthermore, we demonstrate that proinflammatory cytokines produced by Rictor−/− DC after LPS stimulation are key in promoting Th1/Th17 responses. These data establish that mTORC2 activity restraints conventional DC proinflammatory capacity and their ability to polarize T cells following TLR and non-TLR stimulation. Our findings provide new insight into the role of mTORC2 in regulating DC function and may have implications for emerging therapeutic strategies that target mTOR in cancer, infectious diseases, and transplantation.

The mammalian/mechanistic target of rapamycin (mTOR) is a key integrative kinase that functions in two independent complexes, mTOR complex (mTORC)1 and mTORC2. In contrast to the well-defined role of mTORC1 in dendritic cells (DC), little is known about the function of mTORC2. In this study, to our knowledge, we demonstrate for the first time an enhanced ability of mTORC2-deficient myeloid DC to stimulate and polarize allogeneic T cells. We show that activated bone marrow–derived DC from conditional Rictor−/− mice exhibit lower co-inhibitory B7-H1 molecule expression independently of the stimulus and enhanced IL-6, TNF-α, IL-12p70, and IL-23 production following TLR4 ligation. Accordingly, TLR4-activated Rictor−/− DC display augmented allogeneic T cell stimulatory ability, expanding IFN-γ+ and IL-17+, but not IL-10+ or CD4+Foxp3+ regulatory T cells in vitro. A similar DC profile was obtained by stimulating Dectin-1 (C-type lectin family member) on Rictor−/− DC. Using novel CD11c-specific Rictor−/− mice, we confirm the alloreactive Th1 and Th17 cell-polarizing ability of endogenous mTORC2-deficient DC after TLR4 ligation in vivo. Furthermore, we demonstrate that proinflammatory cytokines produced by Rictor−/− DC after LPS stimulation are key in promoting Th1/Th17 responses. These data establish that mTORC2 activity restraints conventional DC proinflammatory capacity and their ability to polarize T cells following TLR and non-TLR stimulation. Our findings provide new insight into the role of mTORC2 in regulating DC function and may have implications for emerging therapeutic strategies that target mTOR in cancer, infectious diseases, and transplantation. The Journal of Immunology, 2015, 194: 000–000.

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by low costimulatory molecule expression, poor T cell stimulatory ability, and Treg expansion (25]), it can also promote DC proinflammatory effects, including enhanced IL-12p70 and impaired IL-10 production (26–29), mediated via augmentation of NF-κB and reduction of STAT-3 activity (26, 27). In contrast, little is known about the function of mTORC2 in APCs. Recently, Brown et al. (30) reported that mTORC2 in mouse DC negatively regulates the inflammatory response through phosphorylation of Akt and cytoplasmic retention of the transcription factor FoxO1 following LPS stimulation. In this study, we have examined the role of mTORC2 in DC in response to different stimuli and in shaping T cell responses.

We report that, compared with control myeloid DC, those lacking mTORC2 exhibit elevated proinflammatory cytokine production, T cell allostimulatory ability, and enhanced capacity to expand IFN-γ- and IL-17-producing T cells without Treg expansion, following TLR4 or Dectin-1, but not TLR2 or CD40 stimulation. Using novel CD11c-specific Rictor−/− mice, we have also demonstrated the Th1 and Th17 cell–polarizing ability of endogenous mTORC2-deficient DC after TLR4 ligation. These novel findings enhance the current understanding of the immunomodulatory function of mTORC2 in DC.

Materials and Methods

Mice

Male C57BL/6 (B6; H-2b), BALB/c (H-2d), and B6.Cg-Tg(Tcra,Tcrb) S21/9 (S21/9) or B6.Cg-Tg(Tcra,Tcrb) S21/9 (S21/9) (9327), p-GSK-3β (k), p-GSK-3β (K), p-GSK-3β (S9) (9336), NF-κB p65 (4764), or NF-κB p65 (S563) (3033). In addition, mAbs to GAPDH or β-actin (Novus Biologicals) were used. After washing, membranes were incubated with HRP-conjugated Abs (Cell Signaling Technology). Band visualization was achieved with SuperSignal West Pico Substrate (Pierce Chemical) and exposure to film. The intensity of individual bands was quantified using ImageJ (NIH) relative to the loading control (β-actin or GAPDH) and represented as relative expression compared with the nonstimulated control band.

DC–T cell cocultures

Allogeneic MLR were performed using γ-irradiated (20 Gy) DC as stimulators and normal CD3+ BALB/c T cells as responders (1:20 DC: T cell ratio). Splenic T cells were isolated by negative selection (11) and labeled with CFSE (Vibrant CFDAse Cell Tracker Kit; Invitrogen). Proliferation was analyzed at day 4 by flow cytometry, and T cell phenotype was determined at day 6. For blocking experiments, anti-mouse IL-23R (10 μg/ml, clone 2A14; Merck) or IL-6 Ab (50 μg/ml, clone MP5-20F3; BD Pharmingen) was added to the MLR at day 0. Ag-specific T cell stimulation was performed using WT or Rictor−/− DC loaded with BALB/c-derived I-Ek(52-68) allopeptide for 16 h and TCR-transgenic (tg) T cells specific for this peptide for 3 d. T cell proliferation was analyzed as described (27).

Stimulation of naive T cells with CD3/CD28 beads

Normal BALB/c T cells were plated in round-bottom 96-well plates (8 × 10^4 cells/well) in either RPMI 1640 complete medium supplemented with rIL-2 (30 U/ml; R&D Systems) or LPS-stimulated control or Rictor−/− DC supernatant (collected 18–20 h poststimulation), in the presence or absence of Dynabeads mouse T-activator CD3/CD28 (1:1 bead-to-cell ratio; Life Technologies). After 6 d, T cells were restimulated for 4 h with PMA/ ionomycin (Sigma-Aldrich) in the presence of Golgi Plugs (BD Biosciences), followed by surface and intracellular staining.

Flow cytometric analyses

For assessment of intracellular cytokine expression, Golgi Plugs and LPS were added to DC for 4–5 h, followed by surface staining for CD11c, treatment with fixation/permeabilization buffer (eBioscence), and intracellular staining. T cells were examined after 4–5 h restimulation with PMA/ionomycin in the presence of Golgi Plugs, performing surface staining, fixation/permeabilization, and intracellular staining with appropriate Ab. DC subsets in spleen were identified by differential expression of CD11c, CD11b, CD80, B220, and PDCA1. Fluorochrome-conjugated mAbs were purchased from eBioscence, BD Biosciences, BioLegend, or Milenyi Biotec. Appropriately conjugated, isotype-matched IgGs served as controls. For phosphoflow analysis, DC were stimulated with LPS (100 ng/ml; Sigma-Aldrich) for 30 min, fixed with 2% paraformaldehyde for 10 min, and permeabilized overnight. Staining was performed with rabbit anti-mouse pAkt S473 or pS6K T389 (Cell Signaling) and donkey anti-rabbit IgG (AF647; Invitrogen). Data were acquired with a LSR II or Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star).

Cytokine quantitation

Cytokines in DC or MLR culture supernatants were quantified by ELISA (eBiosciences) and/or cytometric bead array (CBA; BD Biosciences) where indicated, following the manufacturer’s instructions.

Adaptive transfer of I-Ek TCR tg CD4 T cells

The I-Ek TCR-tg CD4+ T cells (CD90.1*Vβ6*) were purified from lymph nodes and spleens of I-Ek mice by negative depletion, and 5 × 10^5 cells were administered i.v. to CD11c-specific Rictor−/− mice, 1 d after i.p. injection of BALB/c I-Eα-derived allopeptide (I-Eα(52-68)) 500 μg/kg mouse alone or with LPS (100 μg/kg mouse; Sigma-Aldrich). After 5 d, isolated splenocytes were restimulated for 4 h with PMA/ionomycin in the presence of GolgiPlug, followed by surface and intracellular staining.

Statistical analyses

Results are expressed as means ± 1 SD. Significant differences between means were determined using the Student t test or one-way ANOVA test (GraphPad Prism), with p < 0.05 considered significant.

Results

Rictor−/− DC display absence of mTORC2 signaling but intact mTORC1 signaling

We evaluated activation/inhibition of the mTORC1/2 pathway in WT and Rictor−/− DC by immunoblot and phosphoflow analysis. We verified deletion of Rictor in our Rictor−/− DC under different activating conditions (TLR4/2, CD40, or Dectin-1 ligation; Fig. 1A, 1B), whereas total Raptor and mTOR expression remained intact (Fig. 1B). Relative quantification of Western blots showed a slight increase in Raptor expression by Rictor−/− DC after CD40

Allogeneic MLR were performed using γ-irradiated (20 Gy) DC as stimulators and normal CD3+ BALB/c T cells as responders (1:20 DC: T cell ratio). Splenic T cells were isolated by negative selection (11) and labeled with CFSE (Vibrant CFDAse Cell Tracker Kit; Invitrogen). Proliferation was analyzed at day 4 by flow cytometry, and T cell phenotype was determined at day 6. For blocking experiments, anti-mouse IL-23R (10 μg/ml, clone 2A14; Merck) or IL-6 Ab (50 μg/ml, clone MP5-20F3; BD Pharmingen) was added to the MLR at day 0. Ag-specific T cell stimulation was performed using WT or Rictor−/− DC loaded with BALB/c-derived I-Ek(52-68) allopeptide for 16 h and TCR-transgenic (tg) T cells specific for this peptide for 3 d. T cell proliferation was analyzed as described (27).
stimulation, compared with the nonstimulated condition (Fig. 1C).
Interestingly, mTOR expression levels were augmented in non-
stimulated and TLR2-stimulated Rictor \(^{-/-}\) DC compared with
control DC (Fig. 1C). We also analyzed total and phosphorylated
Akt and S6K as markers of mTORC1 and 2 activation, respecti-
vely. Rictor \(^{-/-}\) DC displayed marked reduction of S473-Akt
phosphorylation as expected, whereas T389 phosphorylation of
S6K remained active (Fig. 1B–E), and was slightly increased
compared with control DC, although this was not statistically
significant (Fig. 1C). These results indicate specific inhibition of
mTORC2 in Rictor \(^{-/-}\) DC.

**FIGURE 1.** Rictor \(^{-/-}\) DC show absence of mTORC2 signaling while maintaining mTORC1 signaling. (A) Functions of mTORC1 and mTORC2 in relation to DC activation via TLR, Dectin-1, and CD40. Upon TLR, Dectin-1, or CD40 activation, PI3K or different TNFR-associated factors are recruited, leading to Akt activation, which is affected by mTORC2-mediated Ser\(^{473}\) phosphorylation. Active Akt inhibits the TCS2/1 complex, allowing activation of
mTORC1, which in turn phosphorylates and activates S6K and 4E-BP1. Active Akt also inhibits GSK-3 and FoxO1 function, which translates to regulation of cytokine transcription. Dectin-1 and CD40 signal through different TNFR-associated factors, which can activate Akt, and regulate cytokine transcription via NFAT and NF-\(\kappa\)B. (B) BMDC were generated from WT or Rictor \(^{-/-}\) mice and cultured in the absence (nonstimulated [ns]) or presence of LPS (TLR4), lipoteichoic acid (TLR2), or Curdlan (Dectin 1; Dt1) for 18 h, and then restimulated with the same agents for 30 min before obtaining cell lysates. Total lysates were immunoblotted for the indicated protein. Data are representative of \(n = 3\)–4 independent experiments. (C) All proteins shown were quantified relative to nonstimulated control DC and normalized to \(\beta\)-actin. (D) Phosphorylation levels of Akt S473 and S6K T398 (open profiles) were determined in DCs by flow cytometric analysis following CD11c\(^{+}\) cell purification and LPS stimulation for 1 h. Filled profiles denote samples stained with secondary Ab only. Mean fluorescence intensity (MFI) for pAkt or pS6K is indicated in the *top right corners*. Plots are representative of \(n = 5\) independent experiments. (E) Mean expression of pAkt and pS6K MFI across \(n = 5\) experiments. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\) compared with control DC, and \#\(p < 0.05\) compared with the respective nonstimulated condition. 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; FOXO1, forkhead box O 1; PKC, protein kinase C; Rheb, Ras homolog enriched in brain; S6K, ribosomal protein S6 kinase; SGK1, glucocorticoid inducible kinase-1; TRAF, TNFR-associated factor; TSC1/2, tuberous sclerosis 1/2.

Rictor \(^{-/-}\) DC exhibit reduced size, diminished B7-H1
eexpression, and increased proinflammatory cytokine
production
We found that DC lacking mTORC2 were smaller than WT control
DC (Fig. 2A, 2B), suggesting that mTORC2 may play a role in
regulating DC growth. Also, the yield of DC generated from BM
cells in 7-d culture was significantly lower for Rictor \(^{-/-}\)
compared with control cells (Fig. 2C), indicating that mTORC2 may
regulate DC differentiation. Interestingly, expression of MHC
class II (I-A\(^{\alpha}\)) was increased slightly in Rictor \(^{-/-}\) DC compared
with control DC and especially after Dectin-1 stimulation (3-fold
Unstimulated Rictor knockout DC also showed a modest increase in CD86 (Fig. 2D) and CD80 (data not shown), but significantly diminished levels of CD40 when unstimulated or after CD40 ligation (Fig. 2D). More strikingly, coinhibitory B7-H1 expression was reduced significantly, irrespective of the nature of the stimulus (Fig. 2D). Given that B7-H1 on DC negatively regulates activated T cells and induces Treg (32), this observation suggests that mTORC2-lacking DC have reduced ability to negatively regulate T cell responses.

The accompanying DC cytokine secretion profiles showed significantly increased IL-6, TNF-α, IL-12p70, and IL-23 after TLR4 stimulation compared with control DC (Fig. 2E). These results were confirmed by intracellular staining for each cytokine in DC following TLR4 ligation (Fig. 2F). Rictor knockout DC showed a similar cytokine profile to control DC after TLR2 and CD40 ligation, which resembled the nonstimulated condition (Fig. 2E), except for the significant IL-6 increase after CD40 ligation. In contrast, Dectin-1 stimulation enhanced cytokine secretion in both control and Rictor knockout DC, but only a significant increase in IL-12p70 and IL-23 production by Rictor knockout DC compared with control DC was observed (Fig. 2E).

Next we wanted to investigate the molecules involved in the regulation of the Rictor knockout DC proinflammatory profile. Several authors (30, 33, 34) have reported that Akt is important for downregulation of proinflammatory cytokine production by monocytes/DC. As Brown et al. (30) suggested that mTORC2 in DC reduces the inflammatory response through phosphorylation of Akt and subsequent reduction of phosphorylation/nuclear translocation of the transcription factor FoxO1, we investigated FoxO1 phosphorylation in our experimental setting. As shown in Fig. 3A, S256 phosphorylation of FoxO1 was only slightly inhibited in Rictor knockout DC compared with control DC, mainly following TLR2 stimulation, whereas total FoxO1 was very similar between Rictor knockout DC and control DC. This suggests that FoxO1 may differentially regulate the proinflammatory profile of Rictor knockout DC depending on the stimulus received, and that additional molecules are involved in this regulation. Active Akt has also been shown to be responsible for inducing Ser9 phosphorylation (inhibition) of GSK3-β, which regulates inflammatory cell function.

![Figure 2](https://www.jimmunol.org/)

**Figure 2.** Rictor knockout DC display reduced size and yield, diminished B7-H1 expression, and increased proinflammatory cytokine production. (A) Representative plots of side (SSC) versus forward (FSC) scatter of BMDC and (B) their mean fluorescence intensity (MFI) quantified across n = 7 experiments. (C) DC yield normalized to number of initial cultured BM cells. (D) CD11c+ gated cells were analyzed for I-Ab, CD86, CD40, and B7-H1 expression by flow cytometry in nonstimulated (ns) cultures or those stimulated with LPS (TLR4), lipoteichoic acid (TLR2), CD40L (CD40), or Curdlan (Dectin-1) on day 6 for 18 h. Bars are means + 1 SD of n = 5–8 independent experiments; data normalized to the ns normal DC condition. (E) Cytokine levels in supernatants were assessed by cytokine bead array (IL-6, IL-10, TNF-α) or ELISA (IL-12p70 and IL-23). (F) LPS-stimulated DCs were stained for intracellular cytokines after 5-h culture with LPS and GolgiPlug. Plots show the percentages of CD11c+ gated cells positive for each cytokine in a representative experiment. Data are from n = 4 experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

![Figure 3](https://www.jimmunol.org/)

**Figure 3.** Rictor knockout DC proinflammatory profile is partially regulated by GSK-3. BM-DC were generated from WT or Rictor knockout mice and cultured in the absence (nonstimulated [ns]) or presence of LPS (TLR4), lipoteichoic acid (TLR2), CD40L (CD40), or Curdlan (Dectin 1: D1) for 30 min before obtaining cell lysates. Total lysates were immunoblotted for (A) total and phosphorylated (Ser256) FoxO1, and for (B) phosphorylated GSK-3αβ (S21/9) or GAPDH. Data are representative of n = 2–3 independent experiments.
responses through CREB and NF-κB (33). Taking these data into consideration, we tested phosphorylation of GSK3 in control and Rictor−/− DC. The results showed (Fig. 3B) that Ser3 phosphorylation of GSK3-β is reduced in Rictor−/− DC for all stimuli compared with control DC (Fig. 3B). These data demonstrate that GSK3-β is less inhibited (more activated) in Rictor−/− DC than in control DC. Because GSK3-β regulates cytokine production (33), these results suggest that Rictor−/− DC proinflammatory response is partially regulated by GSK3-β.

TLR4- or Dectin-1–stimulated Rictor−/− DC promote enhanced Th1/Th17 responses, without expanding Treg

The ability of mTORC2 to differentially regulate distinct costimulatory molecules and cytokine production by DC suggested a potential impact on T cell activation. We therefore assessed the capacity of mTORC2-deficient DC to regulate T cell responses. Rictor−/− DC exhibited greater bulk T cell costimulatory ability in MLR following TLR4 ligation (Fig. 4A; Supplemental Fig. 1A), but not after TLR2, CD40, or Dectin-1 stimulation compared with control DC (Fig. 4A). These results were confirmed in an alloantigen-specific setting, where TLR4-activated Rictor−/− DC induced 2-fold enhanced proliferation of Ag-specific TCR-tg T cells as compared with control DC (Fig. 4B).

Next, we investigated T cell subset differentiation in response to Rictor−/− DC stimulation in conventional MLR. Although control DC increased absolute numbers of CD4+CD25+Foxp3+ Treg following TLR2 or Dectin-1 ligation, neither unstimulated nor activated Rictor−/− DC increased alloreactive Treg or T regulatory type-1 cells (CD4+IL-10+) (Fig. 4C). Accordingly, IL-10 levels in MLR supernatants were reduced in the presence of unstimulated or TLR2-stimulated Rictor−/− DC compared with control DC (Fig. 4D). Interestingly, TLR4-activated Rictor−/− DC increased the incidence of IL-6−, IFN-γ−, and IL-17−producing CD4+ T cells (Fig. 4C), compared with those primed by control DC. Consistent with these results, secretion of IFN-γ and IL-17 was augmented significantly when TLR4-stimulated Rictor−/− DC were used as stimulators (Fig. 4D). We also observed a significant increase in IL-6− and IFN-γ−producing T cells by Dectin-1–stimulated Rictor−/− DC (Fig. 4C) with a significant augmentation of secreted IL-17 levels, compared with control DC (Fig. 4D). These results demonstrate an expansion in IFN-γ− and IL-17−secreting T cells by Rictor−/− DC after TLR4 or Dectin-1 stimulation. Th2 cytokines were also examined, and a slight reduction in IL-5–producing T cells after coculture with Rictor−/− DC compared with control DC was observed (Supplemental Fig. 1B).

No differences were observed in IL-4 or TNF-α levels in the culture supernatants (Supplemental Fig. 1C) between control and Rictor−/− DC. Taken together, these results demonstrate that Rictor−/− DC promote enhanced Th1 and Th17 responses in vitro, without expanding Treg, after TLR4 or Dectin-1 stimulation. Thus, mTORC2 negatively regulates the ability of DC to prime Th1 and Th17 cell responses.

Rictor−/− DC-secreted proinflammatory cytokines, principally IL-6 and IL-23, are key to promotion of Th1/Th17 responses

Next, we assessed the mechanism underlying the ability of Rictor−/− DC to induce Th1/Th17 cells. We hypothesized that proinflammatory cytokines produced by Rictor−/− DC (Fig. 2E, 2F) were more likely to promote Th1/Th17 responses than enhanced costimulatory molecule expression by these cells (Fig. 2D). Previously, IL-23 and IL-6 dependence of Th17 differentiation in vivo and its influence on Th1 differentiation have been reported (35–40). Therefore, we investigated the role of Rictor−/− DC-secreted IL-23 and IL-6 in the promotion of Th1 and Th17 cells. Blockade of IL-6, IL-23R, or both molecules in allogeneic MLR markedly reduced the incidence of IFN-γ− and IL-17− CD4+ T cells induced by TLR4-activated Rictor−/− DC (Fig. 5A). Quantification of cytokines se-
Endogenous Rictor<sup>−/−</sup> DC showed increased IL-12p70 and IL-23, and promote T cell proliferation and Th1/Th17 responses after TLR4 stimulation in vivo

Finally, we examined whether endogenous mTORC2-deficient DC could similarly regulate T cell responses in vivo. To address this question, CD11c-specific Rictor<sup>−/−</sup> or control mice were adaptively transferred with I-<sup>E</sup>H3.1 TCR-tg T cells and 2 d later were injected with I-E<sub>D</sub>52-68 peptide, with or without LPS. After 6 d, splenic DC from each group showed similar expression of I-A<sub>D</sub> and CD86, but a significant reduction in B7-H1 expression was observed in mice with Rictor<sup>−/−</sup> DC that received peptide alone, compared with control DC (Fig. 6A). This difference was not observed after administration of peptide and LPS, revealing a difference between splenic DC and in vitro generated BM-derived DCs (BMDCs). We investigated which types of splenic Rictor<sup>−/−</sup> DC showed reduced B7-H1 expression in vivo. Our data show (Fig. 6B, 6C) that only conventional myoid CD11b<sup>+</sup> DC but not the other DC populations exhibited reduced B7-H1 expression with controls, after the in vivo administration of peptide alone. In agreement with BMDC results, splenic Rictor<sup>−/−</sup> DC from animals injected with the allopeptide and LPS exhibited increased production of IL-12p70 and IL-23, compared with controls (Fig. 6D). Splenic 1H3.1 T cells (both percentage and absolute numbers) were expanded significantly in CD11c-specific Rictor<sup>−/−</sup> mice compared with controls (Fig. 7A, 7B), consistent with our in vitro finding of increased T cell proliferation by TLR4-stimulated Rictor<sup>−/−</sup> DC (Fig. 4A, 4B). Splenic 1H3.1 T cells showed significant expansion of IFN-γ- and IL-17-producing T cells, but not IL-10<sup>+</sup>, IL-5<sup>+</sup>, or Foxp3<sup>+</sup> T cells after activation by Rictor<sup>−/−</sup> DC in the presence of LPS (Fig. 7C, 7D). These results confirm our in vitro data (Figs. 2D–F, 4A, 4C) and establish that endogenous mTORC2-deficient DC generate augmented Th1/Th17 responses, while failing to impact Treg, thus supporting a central regulatory role of mTORC2 in DC under inflammatory conditions.

Discussion

Recently, using RAPA and ATP-competitive inhibitors, we defined an immunoregulatory pathway in which RAPA-sensitive mTORC1 in DCs promotes effector T cell expansion and RAPA-insensitive mTORC1 restrains Treg induction (11). In this study, using Rictor<sup>−/−</sup> mice, we investigated the function of mTORC2 in DC under different activating stimuli and also their T cell–polarizing ability. We used both TLR and Dectin-1 ligands, as well as stimulation via CD40 (Fig. 1A) to activate Rictor<sup>−/−</sup> DC and for comparison control DC. We analyzed both TLR4 and TLR2 ligands, which induce signaling through MyD88-Toll/IL-1R domain-containing adapter inducing IFN-β or MyD88, respectively (41). Curdlan is a bacterial product [β-(1,3)-glucan polymer] that acts as a selective Dectin-1 agonist and fully activates DCs, even in the absence of MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β or MyD88, respectively (41). Curdlan is a bacterial product [β-(1,3)-glucan polymer] that acts as a selective Dectin-1 agonist and fully activates DCs, even in the absence of MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β or MyD88, respectively (41). Curdlan is a bacterial product [β-(1,3)-glucan polymer] that acts as a selective Dectin-1 agonist and fully activates DCs, even in the absence of MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β or MyD88, respectively (41). Curdlan is a bacterial product [β-(1,3)-glucan polymer] that acts as a selective Dectin-1 agonist and fully activates DCs, even in the absence of MyD88 and Toll/IL-1R domain-containing adapter inducing IFN-β or MyD88, respectively (41).
and IL-23 production and Th1/Th17 cell polarizing potency. Furthermore, we observed that proinflammatory cytokines produced by Rictor\(^{+/−}\) DC after LPS stimulation were key to promotion of these Th1 and Th17 responses.

As reported for DC exposed to either RAPA (11, 44) or Torin1 (an ATP-competitive dual mTOR inhibitor) (11), mTORC2-deficient DC were smaller and were recovered in reduced yield compared with control DC when propagated from BM cells, suggesting that both mTOR complexes play a role in positively regulating DC generation. By contrast, Wang et al. (45) have reported that Rictor deficiency does not exert strong effects on either DC development or size. These differences may reflect different methods used to generate the DC. Whereas the latter authors used Flt3L, we used GM-CSF and IL-4 to generate DC from mouse BM. DC specifically lacking mTORC2 expressed MHC class II (I-Ab) and costimulatory molecules at similar levels to WT controls, but CD86 and coinhibitory B7-H1 expression were increased and reduced, respectively. Previously, we reported diminished B7-H1 expression by both unstimulated and LPS-stimulated Rictor\(^{+/−}\) DC in vitro (11), and we have now extended these findings to show that reduced B7-H1 expression is also observed after TLR2, CD40, or Dectin-1 stimulation. Furthermore, endogenous Rictor\(^{+/−}\) CD11b+ myeloid DC showed reduced B7-H1 expression compared with WT controls after Ag stimulation in vivo. These findings clearly suggest that B7-H1 expression in these DC populations is positively regulated by mTORC2 activation.

Given that B7-H1 expression on myeloid DC negatively regulates T cell activation and induces Treg (32), our finding that Rictor\(^{+/−}\) DC express reduced levels of B7-H1 is consistent with their enhanced T cell allostimulatory ability. However, we found
that whereas Rictor$^{--}$ DC displayed increased T cell stimulatory ability following TLR4 ligation, this was not apparent after exposure to TLR2, CD40, or Dectin-1 stimulants despite reduced B7-H1 expression. Although there are some contradictory results regarding the T cell stimulatory ability of RAPA-treated DC (11, 46, 47), we have shown (11) that dual mTORC1/2-inhibition (Torin1) in DC reduces their ability to induce T cell proliferation. Thus, our results indicate that mTORC2 expression in DC negatively regulates T cell expansion after TLR4 ligation.

Our data show that Rictor$^{--}$ DC display different cytokine signatures, depending on the activation signal they receive and, accordingly, exhibit T cell–differentiating abilities. When Rictor$^{--}$ DC were stimulated by CD40 ligation, they behaved similarly to unstimulated cells, producing small amounts of cytokines (slight increase in IL-6), and, although promoting IL-2 secretion in allogeneic MLR, T cell responses did not differ from those in control DC-stimulated cultures. When stimulated via TLR2, Rictor$^{--}$ DC produced similar amounts of cytokines compared with CD40 ligation, and in allogeneic MLR, decreased Treg and IL-5–producing T cells, and increased IL-2 production compared with controls, suggesting activation but not skewing of T cells. After TLR4 stimulation, Rictor$^{--}$ DC produced higher levels of IL-12p70, IL-23, IL-6, and TNF-α compared with control DC, consistent with the findings of Brown et al. (30), and expanded T cells that produced IL-6, IFN-γ, and IL-17. We confirmed these observations in vivo by stimulating CD11c-specific Rictor$^{--}$ mice with LPS in an allopeptide-specific setting. DC from these mice produced increased levels of IL-23 and IL-12p70 and expanded Th1 and Th17 cells after LPS stimulation in vivo. In contrast, Dectin1 stimulation of both Rictor$^{--}$ and control DC promoted augmented secretion of all tested cytokines, but only IL-12p70 and IL-23 were significantly increased in Rictor$^{--}$ DC compared with control DC. In MLR, Dectin1-stimulated Rictor$^{--}$ DC expanded IL-6– and IFN-γ-producing T cells, while diminishing Treg. It is known that Dectin-1 stimulation of human DC promotes a Th17 response (48). Accordingly, we observed increased IL-17 production in MLR with Rictor$^{--}$ DC, and expansion of IL-17+ T cells after allogeneic cocultures with both control and Rictor$^{--}$ DC. Taken together, these findings suggest that mTORC2 plays an important role in regulating the inflammatory response after pattern recognition receptor stimulation of DCs. Despite the significant overlap in TLR signaling pathways, Th1/Th17 cell polarizing ability seems to be restricted to TLR4 and Dectin-1 ligation of Rictor$^{--}$ DC.

Our findings led us to investigate the molecules involved in the regulation of the Rictor$^{--}$ DC proinflammatory profile. Akt has been reported to downregulate DC proinflammatory cytokine production (30, 33, 34). In fact, Brown et al. (30) suggest that mTORC2 in DC reduces the inflammatory response through phosphorylation of Akt and cytoplasmic retention of the transcription factor FoxO1 (diminished S256 phosphorylation). We show that, although S473 phosphorylation of Akt is inhibited in Rictor$^{--}$ DC (Fig. 1B–E), S256 phosphorylation of FoxO1 is different between Brown et al. (30) and our results could be due to the different models used. Brown et al. (30) analyzed p-FoxO1 (S256) 6 h after knocking down Rictor by small interfering RNA transfection in DC, whereas we analyzed p-FoxO1 (S256) in DC (Fig. 3A). This difference in accordance, we found that Rictor$^{--}$ DC show a reduced inhibitory action of GSK3-β compared with control DC (Fig. 3B), suggesting that Rictor$^{--}$ DC proinflammatory response is partially regulated by GSK3-β. Whereas GSK3-β may partially regulate this response, there are many other potential molecules that may allow mTOR to regulate the proinflammatory profile of myeloid DC.

Our finding that the cytokine profile of Rictor$^{--}$ DC under different stimulatory conditions is consistent with the T cell fate they promote suggests that the cytokines secreted by these DC play an important role in the observed T cell differentiation. This was verified by culturing T cells with DC supernatants, and
showing an increase in Th1/Th17 cells under the influence of Rictor and DC-secreted cytokines. Many cytokines have been associated with promotion of Th1 and Th17 cells, in particular IL-6, IL-23 (35, 37, 38), and IL-12p70 (51, 52). Blocking two of the cytokines produced by Rictor (IL-23, IL-6, or both) in MLR confirmed their relevance to proliferation of Th1/Th17 cells. However, because other signals apart from IL-6 may also induce IL-23R expression by T cells, our data do not exclude the role of other cytokines in driving T cell differentiation.

These novel findings establish that mTORC2 in conventional DC is important in negatively regulating their proinflammatory activity and ability to promote Th1/Th17 cell responses after TLR4 or Dectin-1 ligation. Our findings provide new insight into the role of mTOR in DC and raise the question of potential therapeutic application. Therefore, mTORC2 selective agonists might be useful in the treatment of immune-mediated inflammation, whereas antagonists might find a place in vaccine development or anti-tumor therapy.

Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1.

TLR4-stimulated Rictor−/− DC promote enhanced T cell proliferation, without expanding Th2 cells. (A) Representative histogram of proliferating T cells cocultured with DC for 4d. Proliferation percentages are shown for each condition. (B) Non-stimulated (ns), TLR4/2−, CD40− or Dectin-1-stimulated DC from WT or Rictor−/− mice were used as stimulators of CD3+ BALB/c T cells in 4d CFSE-MLR. Data show absolute numbers of IL-5-producing CD4+ T cells. Bars are means ± 1SD of n = 3–6 independent experiments. (C) Cytokines in MLR supernatants were assessed by cytokine bead array. Data are from n = 3–4 independent experiments. n.d. = not detected. *P < 0.05, **P < 0.01.