Tuberous sclerosis 1 (Tsc1)-dependent metabolic checkpoint controls development of dendritic cells

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Coordination of cell metabolism and immune signals is crucial for lymphocyte priming. Emerging evidence also highlights the importance of cell metabolism for the activation of innate immunity upon pathogen challenge, but there is little evidence of how this process contributes to immune cell development. Here we show that differentiation of dendritic cells (DCs) from bone marrow precursors is associated with dynamic regulation of mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling and cell metabolism. Unexpectedly, enhancing mTORC1 activity via ablation of its negative regulator tuberous sclerosis 1 (Tsc1) impaired DC development in vivo and in vitro, associated with defective cell survival and proliferation. Moreover, Tsc1 deficiency caused DC spontaneous maturation but a propensity to differentiate into other lineages, and attenuated DC-mediated effector TH1 responses. Blocking mTORC1 activity by rapamycin inhibits DC development and/or maturation, and instead endows DCs with a strong tolerogenic activity to promote T-cell tolerance. However, rapamycin is not an efficient inhibitor of 4EBP1 phosphorylation downstream of mTORC1 activation (30), and may also inhibit mTORC2 activity with prolonged treatment and/or at a high dose (31, 32). To conclusively establish the roles of mTOR in DC development, we have used genetic approaches to eliminate mTOR signaling components, including tuberous sclerosis 1 (Tsc1, a modulator of mTORC1 and mTORC2 activities), Rheb (a key upstream activator of mTORC1), and Rictor (an obligatory component of mTORC2) (15), as well as the downstream effector Myc, alone or in combination, and the most well-established molecular function of mTOR is in protein translation, recent studies have identified an important role of mTOR in activating a metabolic gene-regulatory network via controlling the respective transcription factors in glycolysis and lipid synthesis, HIF1α and SREBP (16). mTOR exists in two complexes, mTORC1 and mTORC2, both of which contribute to T-cell activation and differentiation (17–19). In the innate immune system, mTOR and the upstream PI3K–AKT pathway have a well-established role in modulating the balance between TLR-induced production of pro- and anti-inflammatory DC cytokines, especially IL-12 and IL-10, thereby affecting DC function and immune responses (20–24). Additionally, mTOR signaling promotes the production of type I IFN from plasmacytoid DCs (pDCs) (25), and regulates other cellular events induced by TLR stimulation such as survival of activated DCs (12, 26). These results collectively illustrate an important role of mTOR signaling in the activation of both innate and adaptive immune systems. In contrast, the function of mTOR signaling in the development of DCs is less understood, with many of the findings to date obtained via pharmacological approaches. For instance, blocking mTORC1 activity by rapamycin inhibits DC development and/or maturation, and instead endows DCs with a strong tolerogenic activity to promote T-cell tolerance (19, 27–29). However, rapamycin is not an efficient inhibitor of 4EBP1 phosphorylation downstream of mTORC1 activation (30), and may also inhibit mTORC2 activity with prolonged treatment and/or at a high dose (31, 32). To conclusively establish the roles of mTOR in DC development, we have used genetic approaches to eliminate mTOR signaling components, including tuberous sclerosis 1 (Tsc1, a modulator of mTORC1 and mTORC2 activities), Rheb (a key upstream activator of mTORC1), and Rictor (an obligatory component of mTORC2) (15), as well as the downstream effector Myc, alone or in combination, and the most well-established molecular function of mTOR is in protein translation, recent studies have identified an important role of mTOR in activating a metabolic gene-regulatory network via controlling the respective transcription factors in glycolysis and lipid synthesis, HIF1α and SREBP (16). mTOR exists in two complexes, mTORC1 and mTORC2, both of which contribute to T-cell activation and differentiation (17–19). In the innate immune system, mTOR and the upstream PI3K–AKT pathway have a well-established role in modulating the balance between TLR-induced production of pro- and anti-inflammatory DC cytokines, especially IL-12 and IL-10, thereby affecting DC function and immune responses (20–24). Additionally, mTOR signaling promotes the production of type I IFN from plasmacytoid DCs (pDCs) (25), and regulates other cellular events induced by TLR stimulation such as survival of activated DCs (12, 26). These results collectively illustrate an important role of mTOR signaling in the activation of both innate and adaptive immune systems.

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further complemented them with pharmacological approaches. Contrary to our expectations, deletion of Tsc1 and subsequent mTORC1 activation exerted multiple negative effects on DC development, including survival, proliferation, and lineage differentiation. Loss of Tsc1 up-regulated several metabolic programs including glycolysis, mitochondrial respiration, and lipid synthesis, partly via a Myc-dependent pathway, and this metabolic programming contributed to DC survival and differentiation but not proliferation. Further, Tsc1 signaled through Rheb and mTORC1 to control DC development, whereas mTORC2 activity was largely dispensable in this process. Importantly, DC differentiation is associated with dynamic regulation of glycolytic and lipogenic programs and the accompanying mTORC1 activity. These data point to a unique checkpoint in DC development that is regulated by the interplay between Tsc1-mTORC1-dependent immune signaling and Myc-dependent metabolic programming.

**Results**

**Tsc1 Plays an Important Role in DC Development in Vitro.** DCs originate from hematopoietic stem cells (HSCs) in the bone marrow (BM) through multiple steps of differentiation, and this can be recapitated in the in vitro differentiation system mediated by FLT3L (33–35). To determine the importance of mTORC1 regulation in DC development, we focused on Tsc1, an upstream regulator of mTOR signaling (15), by crossing Tsc1CreER mice with Rosa26-Cre-ERT2 mice (a Cre-ER fusion gene was recombined into the ubiquitously expressed Rosa26 locus) to generate Tsc1CreER/Rosa26-Cre-ERT2 mice (Tsc1CreER mice). To circumvent the detrimental effects of chronic loss of Tsc1 on the function of HSCs (36, 37), we determined the requirement of Tsc1 during DC development through in vitro acute deletion. Specifically, we cultured BM cells with FLT3L in the presence of 4-hydroxytamoxifen (4-OHT), which resulted in efficient deletion of Tsc1 after 2 d of treatment (Fig. S1A). Under these conditions, Tsc1CreER BM cells showed a considerable defect to generate CD11c+ cells (Fig. 1A). Analysis of DC-specific transcripts including Zbtb46, Ccr7, Fth3, and c-Ki (35) in sorted CD11c+ cells confirmed them as DCs (Fig. S1B). The impairment in DC generation was apparent in both conventional DC (cDC; CD11c+B220+) and pDC (CD11c+B220−) subsets (Fig. 1B). Thus, Tsc1 is important for DC development in vitro.

We next determined the temporal requirement of Tsc1 in DC development. First, we assessed the generation of DCs from LinSca-1+c-Ki+ cells (LSKs; a heterogeneous mixture of HSCs and multipotent progenitors) and macrophage and DC precursors (MDPs, one of the earliest precursor populations identified for DCs, defined as Lin−CD11c−Sca-1−CSF1R+ (38–40). Both progenitor cultures lacking Tsc1 were impaired from developing into CD11c+ DCs after FLT3L stimulation (Fig. S1C and D), indicating the involvement of Tsc1 in DC development from multipotent and DC-committed precursors. Second, we cultured BM cells from Tsc1CreER mice with 4-OHT added at various times after the initiation of DC differentiation. Compared with wild-type (WT) control, Tsc1CreER cells were impaired from developing into CD11c+ DCs even when 4-OHT was added at 5 d after the initiation of DC differentiation (Fig. S1E). These results illustrate a direct role of Tsc1 in programming DC development.

**A Cell-Autonomous Role of Tsc1 in the Generation of DCs and Precursors in Vivo.** To determine the role of Tsc1 in DC development in vivo, we treated WT and Tsc1CreER mice with tamoxifen for 5 d and then rested the mice for 5–10 d. Such treatment resulted in efficient deletion of Tsc1 in splenic DCs from Tsc1CreER mice (Fig. S1F). Compared with WT control, Tsc1CreER mice contained a reduced number of cDCs (CD11c−MHC-II+), including both CD8− and CD11b− subsets (Fig. 1C). The pDC (CD11c+ mPDCA-1+) population was also diminished (Fig. 1D). Similar defects in the DC population were observed in mesenteric lymph nodes and nonlabeled organs including liver and kidney (Fig. 1E). Therefore, Tsc1 plays an important role in maintaining DC populations in vivo.

To address whether the reduction of DCs in Tsc1CreER mice was an intrinsic defect, we generated mixed-BM chimeras. Specifically, the recipients (CD45.1.1+) were lethally irradiated and reconstituted with tamoxifen-treated WT or Tsc1CreER BM cells (CD45.1.2+; donor) along with WT BM cells (CD45.1.2−; spike) at a 1:1 ratio. After reconstitution, we analyzed the contributions of donor and spike cells to various immune compartments. Because CD4 T cells are relatively resistant to Tsc1 deletion (41), we used the chimerism of CD4 T cells for normalization (Fig. S1G). DCs, including various subsets, derived from Tsc1-deficient donor cells were greatly underrepresented relative to the spike cells (Fig. 1F). Thus, Tsc1 has a key cell-autonomous role in regulating DC development in vivo. Notably, other myeloid cells lacking Tsc1, such as macrophages and neutrophils, were also decreased in the chimeras (Fig. S1 H and I).
We further determined whether Tsc1 regulates DC development partly by impacting the generation of early precursor MDPs. Following tamoxifen treatment in vivo, the percentage and cell number of MDPs in Tsc1+/CreER mice were significantly decreased compared with those in WT mice (Fig. S2A). Furthermore, more common dendritic precursors (CDPs; defined as Lin−CD11c−Sca-1+CSFR+Flt3+CD117+ and committed precursors of cDCs (preDCs; defined as Lin−IA/E−CD11c+Flt3+CD172a+; 33)) were also reduced upon Tsc1 deletion (Fig. S2 B and C). Associated with the numerical reduction of these precursor populations was a decreased expression of GM-CSFR (CD116), although Flt3 expression was largely normal (Fig. S2D). These data indicate a requirement of Tsc1 in the generation of DC precursors, which may act in synergy with its effect at driving the differentiation from these precursors to mature DCs (Fig. S1D) to promote DC development.

**Tsc1 Promotes DC Survival and Proliferation.** The overall size of DC populations is dependent upon the rates of apoptosis and proliferation. We first evaluated the effects of Tsc1 on the survival of DCs. Caspase activity, a hallmark of apoptotic cell death, was increased in freshly isolated splenic DCs of tamoxifen-treated Tsc1+/CreER mice and of the chimeras reconstituted with Tsc1+/CreER BM cells (Fig. 2 A and B). In vitro FLT3L-derived DCs from 4-OHT–treated Tsc1+/CreER BM cells also showed significantly increased apoptosis as indicated by the loss of the live Annexin V−7-AAD+ population, and this increase was observed when 4-OHT was added at 0, 24, and 48 h after the initiation of DC differentiation (Fig. 2C). These data indicate an important role of Tsc1 in promoting the survival of DCs. We then dissected molecular mechanisms by which Tsc1 regulates DC survival. Bcl-2 family proteins are important regulators of apoptotic cell death, and the balance of anti- and proapoptotic Bcl-2 family members is crucial to dictating the decision between survival and apoptosis (42). The expression of the proapoptotic Bim and Puma was increased in 4-OHT–treated Tsc1+/CreER DCs, whereas the antiapoptotic protein Bel-2 was diminished. In contrast, the activity of p38 MAPK, another important cell death regulator, was comparable between WT and Tsc1-deficient DCs (Fig. 2D). Aside from Bcl-2 family proteins, production of reactive oxygen species (ROS) also contributes to apoptotic cell death (42). Tsc1-deficient DCs produced greatly increased levels of ROS (Fig. 2E). Therefore, the increased apoptosis of Tsc1-deficient DCs is associated with dysregulated Bcl-2 family proteins and elevated oxidative stress.

We next tested the possibility that the decreased DC population in the absence of Tsc1 was also partly ascribed to defective DC proliferation. We labeled freshly isolated BM cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured the cells with FLT3L in the presence of 4-OHT. The proliferation of Tsc1-deficient CD11c+ DCs was similar to that of WT cells when examined at day 3.5. However, the mutant cells showed diminished CFSE dilution when analyzed at day 7.5 (Fig. 2F). Furthermore, a bromodeoxyuridine (BrdU) incorporation assay showed that Tsc1-deficient CD11c+ DCs incorporated less BrdU than did WT cells at day 7.5 (Fig. 2G). Thus, Tsc1 is dispensable for initial DC proliferation but its absence compromises continuous DC proliferation.

**Dysregulated DC Maturation and Functional Differentiation in the Absence of Tsc1.** DCs are the most potent antigen-presenting cells for the activation and differentiation of naïve T cells. To investigate the effect of Tsc1 signaling on DC functions at mediating T-cell responses, we first cocultured WT and Tsc1-deficient DCs with naïve CD4+ T cells from OT-II mice [a T cell receptor (TCR)-transgenic model with T cells recognizing ovalbumin (OVA) amino acids 323–339] in the presence of antigen but no exogenous cytokines to mimic the physiological interaction between DCs and T cells. After 5–6 d, T cells activated with Tsc1-deficient DCs secreted decreased IFN-γ, but similar levels of IL-4, compared with those activated with WT DCs (Fig. 3A). We next used freshly isolated DC subsets from in vivo tamoxifen-treated WT and Tsc1+/CreER mice and cocultured them with OT-II naïve T cells. All DC subsets lacking Tsc1 showed a defective ability to drive T111 but not T12 differentiation (Fig. 3B). Finally, we determined the role of Tsc1 in mediating DC-dependent T-cell responses in vivo. To this end, we generated WT and Tsc1+/CreER BM chimeras and then transferred OVA-specific CD4+ T cells into the chimeras, followed by immunization with the cognate antigen. T cells isolated from Tsc1+/CreER chimera hosts produced less IFN-γ (Fig. 3C). These results demonstrate that Tsc1 signaling in DCs is required for the differentiation of antigen-specific naïve T cells into T11 cells.

To explore the mechanistic basis, we analyzed the effects of Tsc1 deficiency on DC maturation, cytokine production, and lineage differentiation. First, we examined the expression of
various surface markers associated with DC maturation, including CD86, CD80, and CD40. Surprisingly, Tsc1-deficient DCs showed more elevated expression of CD86, CD80, and CD40 than WT cells did (Fig. 3D). Moreover, CD86 was up-regulated on DCs derived from Tsc1-deficient donor cells in the mixed chimeras, although CD80 and CD40 expression in vivo was largely normal (Fig. 3E). Thus, Tsc1 deficiency resulted in spontaneous maturation of DCs, with a more pronounced effect on CD86 up-regulation. However, this was unlikely to cause diminished effector T-cell responses, as these DC molecules generally promote effector responses (43). Second, we examined the expression of proinflammatory cytokines, and found that Il2a (IL-12 p35) was decreased in Tsc1-deficient DCs, whereas the anti-inflammatory cytokine Il10 was comparable between WT and Tsc1-deficient DCs (Fig. 3F). Given the importance of IL-12 for mediating Th1 differentiation (44), the impaired cytokine production from Tsc1-deficient DCs, together with the survival defect and numerical reduction, likely contributed to decreased Th1 responses in responding T cells.

Third, we determined the effects of Tsc1 deficiency on the integrity of DC differentiation. Although CD11c⁺ DCs developed in the absence of Tsc1 expressed normal levels of DC-specific transcripts as described above (Fig. S1A), they aberrantly acquired expression of macrophage and neutrophil markers F4/80 and Ly6G, respectively. This defect was exacerbated with more extended duration of culture (Fig. 3G). To determine whether this altered differentiation was functionally relevant to DC cytokine production, we purified F4/80⁺CD11c⁺ cells and measured their cytokine expression levels. Loss of Tsc1 resulted in a profound down-regulation of Il2a in these populations. In contrast, WT and Tsc1-deficient F4/80⁺CD11c⁺ cells expressed comparable levels of Il2a (Fig. 3H). Therefore, Tsc1 maintains the integrity of DC differentiation, the loss of which results in aberrant up-regulation of other lineage markers and impaired DC cytokine expression.

**Tsc1 Coordinates Cell Growth and Metabolism in DCs.** We next explored the molecular mechanism underlying Tsc1-mediated control of DC development. We noticed that DCs developed in the absence of Tsc1 exhibited an increased cell size. This alteration was evident in BM chimeras (Fig. 4A), indicative of an intrinsic defect. Additionally, cell size was increased in Tsc1-deficient DCs developed from total BM cells, LSKs, or MDPS in vitro (Fig. 4B), and this was also observed when Tsc1 deletion was initiated at 3–5 d after the start of culture (Fig. S3A). Further, the mutant DCs contained more protein and RNA content on a per-cell basis (Fig. S3B). These results indicated that Tsc1-deficient DCs underwent more cell growth. Cell growth is dependent on the regulated expression of amino acid transporters that include CD98 as a key component, and of CD71, the transferrin receptor that mediates iron uptake. Flow cytometry analysis of surface expression of CD98 and CD71 revealed elevated levels of CD98 and CD71 upon loss of Tsc1 (Fig. 4C). This effect was also observed in DCs derived from Tsc1-deficient MDPS (Fig. S4A), indicating an important role of Tsc1 in cell-growth regulation during DC development.

Cell growth is coupled with cell metabolism, which consists of bioenergetic and biosynthetic activities (1, 17). Two major modes of bioenergetics are glycolysis and mitochondrial respiration. To determine the role of Tsc1 in DC metabolism, we first measured the glycolytic activity of WT and Tsc1-deficient cells by the generation of [3-3H]glucose. Compared with FLT3L-derived WT DCs, those lacking Tsc1 displayed up-regulated glycolysis (Fig. 4D). This was verified by the elevated extracellular acidification rate (ECAR) as measured by the Seahorse extracellular flux analyzer (Fig. 4E). Further, mRNA expression of glycolytic enzymes, including Hk2, Ldhα, and Tpi1, as well as Hif1α, the transcription factor that regulates glucose metabolism, were all elevated in DCs derived from Tsc1-deficient BM cells (Fig. 4F) or MDPs (Fig. S4B). Moreover, oxygen consumption rate (OCR), indicative of mitochondrial respiration activity, was increased in Tsc1-deficient cells (Fig. 4G), and this was associated with increased mitochondrial mass (Fig. 4H). Therefore, Tsc1-deficient cells exhibit increased glycolysis and mitochondrial respiration.

Having established an important role of Tsc1 in DC bioenergetics, we next determined whether it also contributes to cell biosynthetic activity. In particular, de novo synthesis of lipids (cholesterol and fatty acids) has been shown to depend upon mTOR activity (45). However, the role of Tsc1 in this process remains unclear because it can either promote or inhibit lipid synthesis in a context-dependent manner (16, 46, 47). We therefore measured the incorporation of [1-14C]acetate into chloroform/methanol-soluble lipids in DCs with normal or absent Tsc1 expression; [1-14C]acetate was used here to bypass the requirement of Tsc1 in glycolysis or mitochondrial respiration.

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** Tsc1 deficiency causes spontaneous maturation but aberrant differentiation of DCs that prevents induction of effector T-cell responses. (A) WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT for 7–8 d and sorted for CD11c⁺ cells, which were then cultured with naïve OT-I T cells in the presence of OVA253-35 and LPS, followed by IFN-γ and IL-4 detection. (B) Freshly isolated splenic DC subsets from tamoxifen-treated WT or Tsc1CreER mice were cocultured with FLT3L and 4-OHT, followed by analysis of CD86, CD80, and CD40 expression on CD11c⁺ cells. (C) Expression of CD86, CD80, and CD40 on splenic DCs from WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT, followed by analysis of CD86, CD80, and CD40 expression on CD11c⁺ and Ly6G expression. (D) WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT, and CD11c⁺F4/80⁺ cells and CD11c⁺F4/80⁺ cells were sorted for RNA analysis. Error bars indicate SEM. Data are representative of two to four independent experiments.
allowing us to directly evaluate the role of Tsc1 in de novo lipid biosynthesis rate (46). Compared with WT DCs, those lacking Tsc1 showed a markedly increased lipid synthesis rate (Fig. 4f). Furthermore, expression of many genes in cholesterol and fatty acid metabolism, including Hmgcs1, Hmgcr, and Scd2, was greatly elevated in DCs derived from Tsc1-deficient BM cells (Fig. 4j) or MDPs (Fig. S4C). Altogether, these results indicate that Tsc1 has a key role in negatively controlling cell growth, bioenergetics, and lipid biosynthesis, thereby coupling cell growth and metabolism.

**The Tsc1–Myc Axis Orchestrates Metabolic Programming of DCs.** The transcription factor Myc is an established regulator of cell metabolism, especially glycolytic activity, and plays a key role in metabolic reprogramming of activated T cells (4). We hypothesized that the increased metabolic activity of Tsc1-deficient DCs is partly dependent upon Myc. Indeed, Myc expression was up-regulated in Tsc1-deficient DC-derived CD11c+ DCs or freshly isolated DCs following in vivo tamoxifen treatment (Fig. 5A). To determine the functional relevance of Myc in Tsc1-mediated control of DC metabolism, we generated *Tsc1lox/lox*Mycfl/fl*Rosa26-Cre-ERT2 (Tsc1/MycCreER) mice. Remarkably, loss of Myc nearly completely blocked elevated glycolysis and de novo lipid synthesis in Tsc1-deficient DCs, even though Myc deficiency alone did not cause major alteration of DC metabolism (Fig. 5 B and C). This rescue effect was associated with the restoration of glycolytic and lipogenic gene expression in DCs deficient in both Tsc1 and Myc (Fig. 5D). Moreover, the increased cell size and CD71 and CD98 expression in Tsc1-deficient DCs were partly rescued by the simultaneous loss of Myc (Fig. 5E). Therefore, the aberrant induction of Myc in Tsc1-deficient DCs accounted, at least partially, for the dysregulated growth and metabolism.

We next evaluated the extent to which Myc-dependent metabolism contributes to DC development. Importantly, the exacerbated apoptosis in Tsc1-deficient DCs was partly blocked by Myc deficiency (Fig. 5F). This blocking was associated with the partial rescue of Bel-2 (Fig. 5G) and Bim dysregulation (Fig. S5A), although ROS production was not corrected (Fig. S5B). In contrast, Myc deletion alone diminished DC proliferation, and DCs developed in the absence of both molecules were still defective in proliferation and total DC production (Fig. 5 H and I). Thus, aberrant induction of Myc contributes to defective survival but not proliferation in Tsc1-deficient DCs. Further, Myc deletion partially blocked the spontaneous maturation of DCs lacking Tsc1, as the increased expression of the maturation markers CD86, CD80, and CD40 in these cells was down-regulated by the deletion of Myc (Fig. 5J). Analysis of BM chimeras further showed that Myc deficiency partially blocked the aberrant up-regulation of CD86 on Tsc1-deficient DCs in vivo (Fig. S5C). Finally, the increased F4/80 and Ly6C expression in the absence of Tsc1 was partly rescued by simultaneous loss of Tsc1 and Myc (Fig. 5K). Taken together, Myc plays a key role in mediating the dysregulated metabolism and contributes to the impaired survival and maturation of Tsc1-deficient DCs. However, other effects, such as the defective proliferation and in vivo differentiation in Tsc1-deficient cells, occur independent of Myc.

**Rapamycin-Sensitive mTORC1 Signaling Contributes to Tsc1-Deficient DC Defects.** We investigated the signaling mechanisms by which Tsc1 controls DC development. Although Tsc1 is known to negatively regulate mTORC1 (15), it also regulates mTORC2 activity (48). Indeed, the defective survival of Tsc1-deficient T cells is insensitive to rapamycin treatment in vitro (49), and has been suggested to be ascribed to diminished mTORC2 activity (50). To determine the biochemical functions of Tsc1 in DCs, we first used immunoblotting to examine canonical targets of mTORC1, namely the phosphorylation of S6, S6K1, and 4EBP1 (15). DCs developed in the absence of Tsc1 showed increased activities of these mTORC1 targets (Fig. 6A). In contrast, the phosphorylation of Akt (Ser473), which is a target of mTORC2, was diminished in the mutant cells (Fig. 6B). Thus, Tsc1 serves as a negative regulator of mTORC1 in DCs but promotes mTORC2 activity. To further examine the role of Tsc1 in restraining mTORC1 activity at the single-cell level, we used flow cytometry to measure phosphorylation of S6 at different time points of DC differentiation. Increased phosphorylation of S6 was observed at 4 d after the initiation of 4-OHT treatment (Fig. 6B), which was largely in agreement with the timing of Tsc1 RNA deletion (Fig. S1A). Moreover, in vivo tamoxifen-treated Tsc1CreER mice had higher levels of S6 activation than WT controls (Fig. 6C). Similarly, DCs derived from Tsc1CreER BM donor cells in mixed chimeras also had increased p-S6 than those derived from WT BM or spike BM cells (Fig. 6D). These results indicate that Tsc1 plays an important role in inhibiting mTORC1 activation in DCs in vitro and in vivo.

To determine whether elevated mTORC1 activation contributes to the defects in Tsc1-deficient DCs, we treated BM cells in vitro with the mTORC1 inhibitor rapamycin. As expected, rapamycin blocked the elevated mTORC1 activity in Tsc1-deficient DCs.

![Fig. 4. Loss of Tsc1 up-regulates cell growth and multiple metabolic programs including glycolysis, mitochondrial respiration, and lipid biosynthesis. (A) Cell size of splenic DCs from spike BM and WT or Tsc1CreER donor BM-derived cells in the mixed chimeras (generated as described in Fig. 1F), FSC, forward scatter. (B) Size of CD11c+ cells derived from 4-OHT–treated WT or Tsc1CreER total BM cells (Left), LSKs (Center), or MDPs (Right). (C–J) BM cells from WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT, followed by analysis of CD71 and CD98 expression (C), glycolytic activity through measurement of the generation of [3H]2O from [3-3H]glucose (DPM, disintegrations per minute) (D), extracellular acidification rate (m pH, milli-pH units) (E), mRNA expression of glycolysis-related genes (F), oxygen consumption rate (g), mitochondria mass as determined by MitoTracker Green (H), de novo lipogenesis assay (I), and mRNA expression of lipogenic genes (J). Error bars indicate SEM. Data are representative of two to five independent experiments.](#)
DCs (Fig. 6F). Whereas rapamycin treatment had a modest effect in reducing DC production from WT cells, it largely restored the percentage and total number of Tsc1-deficient DCs (Fig. 6F). Rapamycin also blocked excessive expression of CD86, CD80, and CD40 (Fig. 6G) and rescued the aberrant induction of F4/80 and Ly6G in the mutant cells (Fig. 6H). At the cellular and molecular levels, rapamycin blocked the up-regulation of apoptosis, cell size, ROS production, and mitochondrial mass (Fig. 6I), as well as Myc expression (Fig. 6J), as observed in Tsc1-deficient DCs. Taken together, the increased mTORC1 activity is required to drive the developmental and metabolic defects of Tsc1-deficient DCs.

One caveat remains, however, because the extended treatment of rapamycin can also inhibit mTORC2 activity (31, 32). To determine the effects of mTORC2 on DC development, we used a similar strategy as Tsc1 deletion and generated Rictor alleles and Rosa26-CreER T2 mice (RictorCreER2) which allowed us to acutely delete Rictor, the mTORC2-defining component. BM cells with deletion of Rictor in vitro developed normally into DCs, although total DC number trended lower (Fig. S6A). This finding is in agreement with a recent study showing a dispensable role of Rictor in GM-CSF-derived DC development (29). The mutant DCs showed comparable cell death, cell size, and expression of DC maturation markers as WT cells (Fig. S6B–D). Therefore, deficiency of Rictor did not alter DC development, suggesting that loss of mTORC2 activity in Tsc1-deficient DCs is insufficient to drive the disrupted immune homeostasis. These results collectively indicate that aberrant activation of mTORC1 largely accounts for impaired DC development.

**Tsc1 Signals Through RhoA to Control mTORC1 and DC Development.**

One of the important inputs to mTORC1 is mediated by the small G protein Rheb, although Rheb-independent pathways have also been identified (17). For example, the kinase AMPK can directly phosphorylate Raptor to mediate a metabolic checkpoint (31). Having established a role for Tsc1 in DC development via mTORC1 inhibition, we next determined whether this effect is mediated by Rheb. To this end, we used the Rosa26-CreER T2 system and generated mice lacking both Tsc1 and Rheb (Tsc1/RhebCreER2). We cultured BM cells from the mutant mice and proper controls with FLT3L and examined DC generation following acute deletion in vitro. The diminished generation of DCs in the absence of Tsc1 was restored to WT levels by the simultaneous loss of Rheb (Fig. 7A). We then determined the effects of Rheb deficiency on the altered apoptosis and proliferation of Tsc1-deficient DCs. The increased apoptosis of Tsc1-deficient DCs was nearly completely restored in Tsc1/Rheb-deficient DCs (Fig. 7B), associated with the reversal of ROS overproduction and dysregulated expression of Bcl-2 family proteins (Fig. 7C and D). Additionally, the defective proliferation of Tsc1-deficient DCs was similarly rescued by the loss of Rheb (Fig. 7E). Furthermore, the altered maturation markers as observed in Tsc1-deficient DCs were restored to WT levels in Tsc1/Rheb-deficient cells (Fig. 7F). These results indicate that Tsc1 signals to mTORC1 inhibition in a strictly Rheb-dependent manner. Surprisingly, deficiency of Rheb alone did not have a strong effect on DC development, survival, or proliferation (Fig. 7A–F). Thus, whereas an excessive Rheb-mTORC1 activity is detrimental to DC generation, loss of function of this pathway appears to be compatible with DC development.

Given the importance of cell growth and metabolism in DC development, we further measured the role of the Tsc1–Rheb axis in this process. The increased cell growth and nutrient receptor expression in Tsc1-deficient DCs were blocked by the simultaneous
loss of Rheb (Fig. 7G). Further, the elevated mRNA expression of glycolytic (Hk2, Ldhα, and Tpi1) and lipogenic genes (Hmgcs1, Hmgcr, and Scd2), as observed in Tsc1-deficient DCs, was completely restored in Tsc1/Rheb-deficient cells (Fig. 7H). Finally, Rheb deficiency restored the aberrant induction of p-S6 and p-4EBP1 observed in Tsc1-deficient cells (Fig. 7I and J).

We next explored how mTORC1 activity is regulated during DC development. We cultured BM cells with FLT3L and examined phosphorylation of S6 and 4EBP1 at different time points. Phosphorylation of these molecules reached a high level at day 5 and then abated (Fig. 7K). Importantly, the cell size and glycolytic and lipogenic activities followed a similar biphasic pattern of regulation, with the highest levels observed at days 3–5 (Fig. 7L–N). Therefore, mTORC1 activity and mTORC1-dependent metabolism are dynamically regulated during DC development, whereas uncontrolled mTORC1 activation impairs DC development (Fig. 7O).

Discussion

A fundamental question in immunology is how the development of DCs is regulated by cellular and molecular processes. The recent identification of distinct DC precursors and transcription factors orchestrating differentiation of DCs and various subsets has revolutionized our understanding of DC biology (33–35). In contrast, we have limited information on how intracellular signaling networks program DC development. Moreover, whereas emerging evidence highlights a role of cell metabolism in the activation of innate and adaptive immunity (1–3), its involvement in the development of immune cells, and in particular DCs, remains undefined. Here we describe that the intersection between Tsc1-mTORC1 signaling and Myc-dependent metabolism orchestrates a previously unappreciated metabolic checkpoint to control DC development. Surprisingly, loss of Tsc1 and the ensuing mTORC1 up-regulation disrupt development of DCs by impairing their survival, proliferation, and differentiation. These defects impair T helper 1 effector responses, despite the elevated expression of maturation markers. Mechanistically, Tsc1 controls DC development in part by actively repressing Myc-mediated metabolic programs, especially glycolysis and lipid synthesis. This process requires Tsc1 to signal through Rheb to inhibit mTORC1 activity. Our results therefore identify Tsc1-Rheb-mTORC1 as a central pathway to program DC development by mediating the interplay between immune signaling and Myc-mediated metabolic programs.

Emerging evidence from mouse genetic models highlights a role of mTOR in DC development and activation. Specifically, deletion of Pten facilitates FLT3L but not GM-CSF–driven DC development in vitro and the expansion of CD8+ DCs in vivo (27). Further, deficiency of Tsc1 has been recently shown to impair TLR–induced activation and maturation of DCs derived from GM-CSF; although Tsc1 loss does not affect DC development in response to GM-CSF (52) or DC terminal differentiation or maintenance (27). In contrast, our results have identified a detrimental effect of Tsc1 deficiency on the generation of DCs in vivo and in response to FLT3L stimulation in

![Fig. 6. Rapamycin-sensitive mTORC1 signaling contributes to Tsc1-deficient DC defects.](image)

(A) BM cells from WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT, followed by immunoblot analysis of p-S6, p-56k, p-4EBP1, and p-AKT (Ser473) in purified CD11c+ cells. (B) BM cells from WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT, followed by flow cytometry analysis of p-S6 at the indicated time points of DC differentiation. (C and D) Phosphorylation of S6 was determined in splenic DCs from tamoxifen-treated WT or Tsc1CreER mice (C) or from WT or Tsc1CreER donor BM-derived cells (Left) and spike BM cells (Right) in the mixed chimeras (generated as described in Fig. 1A). (E–J) BM cells from WT or Tsc1CreER mice were cultured with FLT3L and 4-OHT in the presence of rapamycin or control, followed by analyses of phosphorylation of S6 (E), CD11c+ DC percentage (Left) and number (Right) (input BM cells, 2 × 10^5) (F), maturation marker expression (G), F4/80 and Ly6G expression (H), Annexin V staining, cell size, ROS production, and mitochondrial mass (J), and Myc expression (the numbers below the lanes indicate the band intensity relative to that of the loading control β-actin) (J). Error bars indicate SEM. Data are representative of two to four independent experiments.
vitro, thereby highlighting an intrinsic role of Tsc1 in DC development. The simplest interpretation is that the mTOR regulators exert pathway- and context-specific regulation of DC biology. For instance, Pten is an inhibitor of both mTORC1 and mTORC2 activities by repressing upstream PI3K activity (17). Pten also has functions independent of PI3K/mTOR, such as acting in the nucleus to maintain chromosomal stability (53). In contrast, loss of Tsc1 up-regulates mTORC1 but diminishes mTORC2 activity, although the reduction of mTORC2 activity alone (as achieved by Rictor deletion) is not sufficient to alter DC development. Consistent with a dominant role of mTORC1 in this process, the defect in Tsc1-deficient DCs can be rescued by rapamycin and, more importantly, by Rheb deficiency. Furthermore, deletion of Pten but not Tsc1 using CD11c-Cre mice affects homeostasis of DCs (27). These results collectively support an important but complex role of mTORC1 signaling in DC development.

Aside from a reduction in overall DC populations, DCs that were able to develop in the absence of Tsc1 (as indicated by the normal expression of DC-specific transcripts) aberrantly up-regulate markers for macrophages and neutrophils. These results indicate that Tsc1 deficiency results in a loss of DC developmental integrity by failing to repress diversion into alternative lineages. Further, these mutant DCs undergo spontaneous maturation, as shown by the excessive induction of costimulatory molecules, especially CD86, that was obvious in Tsc1-deficient DCs in vitro and in vivo. This finding is in agreement with the observation that rapamycin treatment during DC development reduces DC maturation (19, 28, 29), thereby providing genetic evidence for a role of mTORC1 in promoting DC maturation. Despite the increased CD86 expression, DCs developed in the absence of Tsc1 were unable to effectively mediate the differentiation of Tfh cells, and such a functional loss is associated with aberrant lineage development. Therefore, the aberrant differentiation of Tsc1-deficient DCs, likely acting in synergy with the survival defect and numerical reduction, contributes to the loss of DC functional fitness, further highlighting the importance of active control of mTORC1 activity for DC development and function.

We further link Tsc1-mTORC1 functions to metabolic programming of DC development. Remarkably, loss of Tsc1 results in extensive changes of multiple metabolic programs. First, DCs developed in the absence of Tsc1 up-regulate glycolysis and expression of key glycolytic enzymes. Second, the mutant cells show increased mitochondrial metabolism as shown by increased rate of mitochondrial respiration, excessive ROS production, and elevated mitochondrial mass. Third, lipid synthesis is markedly increased as well, associated with up-regulated genes in both fatty acid and cholesterol metabolic pathways. Further, the dysregulated metabolism in Tsc1-deficient cells is contingent upon Rheb function, thereby highlighting an important role of Tsc1-Rheb-mTORC1 to coordinately regulate metabolic programs during DC development. However, DC development proceeds normally in the absence of Rheb, suggesting the presence of a Rheb-independent pathway (e.g., the compensation from the homolog Rhebl1/Rheb2 (54) or a distinct GTPase that is yet to be identified) to mediate mTORC1 activation during early DC development. Importantly, expression of the key metabolic transcription factor Myc is enhanced in Tsc1-deficient cells, and deletion of Myc has a marked effect to block the dysregulated metabolism and also partly rectifies abnormal DC survival and maturation. These results point to a unique metabolic check-
point mediated by the interplay between Tsc1-Rheb-mTORC1 signaling and Myc-dependent bioenergetic and biosynthetic activities that actively orchestrate DC development. Our study has established a key metabolic checkpoint in immune cell development, and this is consistent with the recent identification of an Lkb1-dependent but mTORC1-independent metabolic control mechanism required for HSC development (55–57).

Despite the general role of mTORC1 and metabolism in promoting cell proliferation, we had an unexpected finding that DCs developed in the absence of Tsc1 were impaired in continuous proliferation whereas the initial proliferation was normal. Additionally, this effect was independent of Myc. In fact, deficiency of Myc alone reduced DC proliferation. Although the precise mechanism remains to be established, we noticed that deletion of another mTOR inhibitor, Pten, depletes HSCs by inducing the expression of p53 and other tumor suppressors (58). We speculate that the aberrant cell growth and metabolism due to mTORC1 hyperactivation in the absence of Tsc1 induces metabolic stress that, once accumulated, leads to cell-cycle arrest and apoptosis induction, possibly via a tumor suppressor response. Future work is warranted to identify the molecular components involved.

In summary, we have identified a unique metabolic checkpoint crucial for DC development (Fig. 7O). Although emerging evidence highlights a role of glucose metabolism in the activation of innate immunity, especially in response to TLR stimulation (11–14), whether cell metabolism contributes to DC development remains unclear. Similarly, whereas the role of mTOR signaling, including the Tsc1/Tsc2 pathway, in TLR responses in macrophages, DCs, and other cells is beginning to be recognized (20, 52, 59), its involvement in the development of DCs has not been appreciated. Given the evolutionarily conserved function of mTOR signaling, we speculate that mTORC1-dependent metabolic programming of DC development represents a previously unappreciated paradigm of metabolic control of immune cell development that can be applied to the generation of other myeloid cells and additional immune lineages.

Materials and Methods

Mice and BM Chimeras. C57BL/6, CD45.1, Thy1.1, and OT-II mice were purchased from The Jackson Laboratory. Tsc1fllox/flox, Mycflox/flox, Rhebfllox/flox, and Rictorfllox/flox mice were bred with Rosa26-Cre-ERT2 mice, and have been backcrossed to the C57BL/6 background for at least eight generations. WT controls were Cre+ mice in the same genetic background to account for Cre effects. For in vivo tamoxifen treatment, WT or Tsc1creERT2 (CD45.2.2+) mice were mixed with Rosa26-Cre-ERT2 (CD45.2.2+) mice and were treated with tamoxifen (11 Gy) CD45.1.1 mice, as described previously (60). For complete BM experiments, BM cells from tamoxifen-treated WT or Tsc1creERT2 CD45.2.2+ mice were transferred into lethally irradiated CD45.1.1.1 mice. Animal protocols were approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital.

Cell Purification and Cultures. Mouse spleens were digested with Collagenase D (Roche), and DCs (CD11c+CD19-DX5), CD8+ T cells (CD3+CD8+), and CD11b+ DCs (CD11c+CD19-CD120a-CD19-CD1D-CD8+DX5) were isolated by negative selection using MACS. Nonlymphoid organs were isolated as described (61). Lympocytes were sorted for naïve T cells (CD4+CD62L+CD69-CD44-CD25-) and transferred into the culture. Tsc1−/− cells, Myc−/− cells, and pDCs (CD123+CD11c-PDLA-1+CD19-CD15X) were sorted on a Flow Cytometer (BD FACS Aria). CD11c+ cells were cultured in RPMI-1640 medium containing 10% (vol/vol) FBS and mouse IL-7 (20 ng/mL) and recombinant murine IL-4 (50 U/mL) and stimulated with anti-CD40 (CD154) (5 μg/mL) for 3 days. The bioenergetic activities of the ECAR and OCR pathways were measured using the Seahorse XF24-3 Extracellular Flux Analyzer (Seahorse Bioscience). For the measurement of ATP production, cells were cultured in RPMI-1640 medium containing 10% (vol/vol) FBS and mouse IFN-γ (200 U/mL) for 24 h. ATP concentration was measured using a luciferase assay kit (MTL BioSolutions). Mitochondrial membrane potential was measured using a mitochondrial membrane potential kit (Sigma). To measure mitochondrial DNA content, cells were treated with RNase and DAPI for nuclear staining. The DNA content was analyzed by flow cytometry. For flow cytometric analysis, lymphocytes were incubated with FITC annexin V and PI (Molecular Probes) for 15 min at 4 °C according to the manufacturer’s instructions (BD Biosciences). The cycling threshold value of the endogenous control gene (HPRT or actin) was subtracted from the cycling threshold value of each target gene to generate the change in cycling threshold (ΔCT). The relative expression of each target gene is expressed as the fold change relative to that of WT samples (2−ΔΔCT), as described (61, 62), and the values of biological replicates are presented. Immunoblotting was performed as described (61, 62) using the following antibodies: p-30kDa Akt (sc-7272-P), p-40kDa Akt (sc-7245-P), p-44kDa ERK (sc-7489-P), p-38kDa MAPK (sc-12757-P), p-ERK (sc-13270-P), and β-actin (both from Sigma). Protein concentration was measured by the BCA Protein Assay Kit (Thermo Scientific Pierce), and RNA concentration was measured using a NanoDrop spectrophotometer.

Statistical Analysis. P values were calculated using Student t test or ANOVA (GraphPad Prism). P values of less than 0.05 were considered significant. All error bars in the graphs represent SEM.

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18. Powell JD, Pollizzi KN, Heikamp EB, Horton MR (2012) Regulation of immune re- 
Supporting Information
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**Fig. S1.** Tsc1 plays an important role in dendritic cell (DC) generation. (A) WT or Tsc1<sub>CreER</sub> bone marrow (BM) cells were cultured in the presence of FLT3L and 4-hydroxytamoxifen (4-OHT), and Tsc1 deletion was detected by real-time PCR at the indicated time points. (B) BM cells from WT or Tsc1<sub>CreER</sub> mice were cultured with FLT3L and 4-OHT, followed by analysis of RNA expression of the indicated genes. (C and D) The progenitor populations of Lin<sup>−</sup>Sca-1<sup>−</sup>c-Kit<sup>+</sup> (LSK) cells (C) and macrophage and DC precursors (MDPs) (D) were isolated from WT or Tsc1<sub>CreER</sub> mice and cultured with FLT3L and 4-OHT for 6 d, followed by analysis of CD11c<sup>+</sup> DC percentage and production. (E) WT or Tsc1<sub>CreER</sub> BM cells (input BM cells, 2 × 10<sup>6</sup>) were cultured with FLT3L, with 4-OHT added at days 0, 1, 3, and 5, followed by measurement of CD11c<sup>+</sup> DC number at day 10. (F) WT or Tsc1<sub>CreER</sub> mice were treated with tamoxifen for 5 d, followed by resting for 5–10 d, and deletion of Tsc1 in splenic DCs was determined. (G–I) Analysis of CD4<sup>+</sup> cells (G), macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>; H), and neutrophils (CD11b<sup>+</sup>Ly6G<sup>+</sup>; I) in the mixed-BM chimeras. Error bars indicate SEM. Data are representative of two or three independent experiments.
**Fig. S2.** Tsc1 is required for the generation of DC precursors in vivo. WT or Tsc1\textsuperscript{CreER} mice were treated with tamoxifen in vivo for 5 d, followed by resting for 5–10 d. (A) Flow cytometry of MDPs (Lin\textsuperscript{−}Sca-1\textsuperscript{−}CSF1R\textsuperscript{+}) in the BM of WT or Tsc1\textsuperscript{CreER} mice. (Right) Cell numbers of MDPs. (B and C) Flow cytometry of CDPs (Lin\textsuperscript{−}CD11c\textsuperscript{−}Sca-1\textsuperscript{−}CSF1R\textsuperscript{+}Flt3\textsuperscript{+}CD172\textalpha\textsuperscript{int}) (B) and precDCs (Lin\textsuperscript{−}MHCI\textsuperscript{−}CD11c\textsuperscript{+}Flt3\textsuperscript{+}CD172\textalpha\textsuperscript{int}) (C) in the BM of WT or Tsc1\textsuperscript{CreER} mice. (Lower) Cell numbers of CDPs or precDCs. (D) Expression of CD116 and Flt3 on MDPs and CDPs in the BM of WT or Tsc1\textsuperscript{CreER} mice. Error bars indicate SEM. Data are representative of two or three independent experiments.

**Fig. S3.** Tsc1 mediates dynamic regulation of DC growth. (A) WT or Tsc1\textsuperscript{CreER} BM cells were cultured with FLT3L, with 4-OHT added at days 0, 1, 3, and 5, followed by analysis of expression of cell size of CD11c\textsuperscript{+} DCs at day 10. FSC, forward scatter. (B) WT or Tsc1\textsuperscript{CreER} BM cells were cultured in the presence of FLT3L and 4-OHT, followed by analysis of protein and RNA concentrations of CD11c\textsuperscript{+} DCs at day 5.5. Error bars indicate SEM. Data are representative of two independent experiments.
Fig. S4. DCs derived from Tsc1-deficient MDPs show up-regulated cell metabolism. MDPs from WT or Tsc1\textsuperscript{CreER} mice were cultured with FLT3L and 4-OHT for 6 d, followed by analysis of CD11c\textsuperscript{+} DCs for CD71 and CD98 expression (A) and mRNA expression of glycolytic (B) and lipogenic genes (C). Error bars indicate SEM. Data are representative of one experiment.

Fig. S5. Myc dysregulation contributes to impaired survival and maturation of Tsc1-deficient DCs. (A and B) BM cells from WT, Tsc1\textsuperscript{CreER}, Myc\textsuperscript{CreER}, or Tsc1/Myc\textsuperscript{CreER} mice were cultured with FLT3L and 4-OHT, followed by analysis of Bim expression (A) and reactive oxygen species (ROS) production (B). (C) Expression of CD86 on splenic DCs from WT, Tsc1\textsuperscript{CreER}, Myc\textsuperscript{CreER}, or Tsc1/Myc\textsuperscript{CreER} complete chimera mice. Data are representative of two independent experiments.

Fig. S6. Deficiency of Rictor does not exert strong effects on DC development. WT or Rictor\textsuperscript{CreER} BM cells (input BM cells, 3 × 10\textsuperscript{6}) were cultured with FLT3L and 4-OHT, followed by analysis of CD11c\textsuperscript{+} percentage (Left) and cell number (Right) (A), Annexin V and 7-AAD staining (B), cell size (C), and maturation marker expression (D). Error bars indicate SEM. Data are representative of two independent experiments.