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Critical Role of the Tumor Suppressor Tuberous Sclerosis Complex 1 in Dendritic Cell Activation of CD4 T Cells by Promoting MHC Class II Expression via IRF4 and CIITA

Hongjie Pan,* Thomas F. O’Brien,*† Gabriela Wright, ‡ Jialong Yang,* Jinwook Shin,* Kenneth L. Wright,‡ and Xiao-Ping Zhong*†

Dendritic cell (DC) maturation is characterized by upregulation of cell-surface MHC class II (MHC-II) and costimulatory molecules, and production of a variety of cytokines that can shape both innate and adaptive immunity. Paradoxically, transcription of the MHC-II genes, as well as its activator, CIITA, is rapidly silenced during DC maturation. The mechanisms that control CIITA/MHC-II expression and silencing have not been fully understood. We report in this article that the tumor suppressor tuberous sclerosis complex 1 (TSC1) is a critical regulator of DC function for both innate and adaptive immunity. Its deficiency in DCs results in increased mammalian target of rapamycin (mTOR) complex 1 but decreased mTORC2 signaling, altered cytokine production, impaired CIITA/MHC-II expression, and defective Ag presentation to CD4 T cells after TLR4 stimulation. We demonstrate further that IFN regulatory factor 4 can directly bind to CIITA promoters, and decreased IFN regulatory factor 4 expression is partially responsible for decreased CIITA/MHC-II expression in TSC1-deficient DCs. Moreover, we identify that CIITA/MHC-II silencing during DC maturation requires mTOR complex 1 activity. Together, our data reveal unexpected roles of TSC1/mTOR that control multifaceted functions of DCs. The Journal of Immunology, 2013, 191: 699–707.

Dendritic cells (DCs) are professional APCs specialized for the initiation of immune responses and they provide the key link between the innate and adaptive immune systems. They are divided into two major subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). cDCs exist in two functionally and phenotypically distinct states: immature and mature. The immature cDCs are highly active at all forms of endocytosis; they express low levels of MHC class II (MHC-II) and costimulatory molecules such as CD40, CD80, and CD86 at the cell surface (1, 2). Inflammatory stimuli such as exposure to pathogens trigger an irreversible DC maturation process that is accompanied by increased production of cytokines and expression of costimulatory molecules and MHC-II on the cell surface. DC maturation ensures effective induction of adaptive immune responses through presenting Ags by MHC molecules and providing costimulation to T cells (3–5).

MHC-II expression in DCs is dynamically regulated by multiple mechanisms. Among those, CIITA is essential for MHC-II transcription by formation of a multiple component transcription activation complex (6–11). In immature cDCs, CIITA is actively transcribed, leading to high levels of MHC-II mRNA expression. However, immature DCs maintain low levels of MHC-II protein at the cell surface in the steady-state because of ubiquitination of the MHC-II β-chain, which leads to rapid internalization of the MHC-II protein to the endosomal compartment. Upon DC maturation after TLR stimulation, MHC-II ubiquitination is rapidly decreased (12–15), allowing translocation of MHC-II to the cell surface for Ag presentation. Concurrently, during DC maturation, CIITA is rapidly silenced at the transcription level (16). The down-regulation of CIITA ensures silencing of new MHC-II transcription in mature DCs (17). Silencing of MHC-II gene in mature DCs has been proposed to allow temporal “fix” of microbial peptide–MHC-II complexes expressed on the DC surface to promote specific anti-microbial T cell responses. CIITA repression in mature DCs is known to involve changes in histone acetylation across the gene locus and specific binding of positive regulatory domain 1 (PRDM1) to the promoters (16, 17). However, the mechanisms that trigger CIITA–MHC-II silencing during DC maturation is unknown. Recent studies have revealed that the mammalian target of rapamycin (mTOR), a serine-threonine kinase that acts as a central regulator for protein synthesis and cell growth, plays important roles in innate immunity (18). mTOR forms two signaling complexes, mTOR complex 1 (mTOR1) and complex 2 (mTOR2), with distinct signaling properties. The mTOR1 consists of mTOR, raptor, and mLST8, whereas the mTOR2 contains mTOR, rictor, and mLST8. mTOR1 phosphorylates pS6K1 and 4E-BP1 to promote cell growth and proliferation, and is sensitive to rapamycin inhibition. mTOR2 phosphorylates Akt on S473, PKCα, and PDK1 to regulate cell survival, actin polymerization, and Th2

*Department of Pediatrics-Allergy and Immunology, Duke University Medical Center, Durham, NC 27710; †Department of Immunology, Duke University Medical Center, Durham, NC 27710; and H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL 33612

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Address correspondence and reprint requests to Dr. Xiao-Ping Zhong, Room 133 MSRB-I, Research Drive, Department of Pediatrics-Allergy and Immunology, Box 2644, Duke University Medical Center, Durham, NC 27710. E-mail address: zhong001@mc.duke.edu

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Abbreviations used in this article: BM, bone marrow; BMDC, bone marrow–derived dendritic cell; cDC, conventional dendritic cell; ChIP, chromatin immunoprecipitation; DC, dendritic cell; IRF, IFN regulatory factor; KO, knockout; MHC-II, MHC class II; mTOR, mammalian target of rapamycin; mTOR1, mTOR complex 1; pDC, plasmacytoid DC; qRT-PCR, quantitative RT-PCR; shRNA, short hairpin RNA; TSC, tuberous sclerosis complex; WT, wild-type.

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immune response, respectively (19, 20). Although studies of mTOR
deficiency in the innate immune cells have not been reported, inhi-
bition of mTORC1 by rapamycin can influence cytokine pro-
duction after TLR stimulation (21, 22). Furthermore, defects of
effector molecules downstream of mTOR have profound impacts on
innate immunity. Deficiency of p66Shc2 substantially decreases
TLR- or virus-induced IFN-α production by pDCs (23). In contrast,
deficiency of 4E-BP1/2, which suppress translation initiation, causes
enhanced IFNα and IFNβ production and resistance to viral infec-
tion in vitro and in vivo because of increased IRF-7 translation (24).
The tuberous sclerosis complex 1 (TSC1) is a tumor suppressor
that associates with TSC2 to form a heterodimer. TSC1 stabilizes
TSC2 by preventing ubiquitin-mediated degradation (25). TSC2/1
complex inhibits Rheb, a small GTPase protein that promotes
mTORC1 activation (26). Although emerging evidence indicates
that TSC1 is a critical regulator in multiple cell lineages within the
innate system (27–35), its role in DCs to control adaptive immune
responses is unclear. In this study, we demonstrate that mTORC1 is
critical for MHC-II silencing during DC maturation. TSC1 inhibits
mTORC1 activation in DCs to ensure MHC-II expression on DCs,
which is required for Ag presentation and CD4 T cell activation.
We further reveal that TSC1 through mTORC1 promotes expression
of IRF4, which directly binds to the CITA promoters in DCs,
resulting in CITA induction and subsequent MHC-II expression.

Materials and Methods

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**Mice and reagents**

* TSC1-f/f-ERCre mice were previously described (31, 36, 37). CD11cCre mice were mated with the Jackson Laboratory (38). Mice were i.p. injected with 200 μl of 10 mg/ml tamoxifen on days 1, 2, and 5, followed by bone marrow (BM) harvesting on day 8. OTII TCR transgenic mice and C57BL/6 mice were purchased from The Jackson Laboratory. All animals were housed in specific pathogen-free conditions. Experiments described were approved by the Institutional Animal Care and Use Committee of Duke University.

**Generation of BM-derived DCs**

BM cells from femurs and tibias were flushed and plated into Petri dishes containing RPMI 1640 supplemented with 10% FBS, 10 mM HEPES pH 7.0, 100 U/ml penicillin, 100 U/ml streptomycin, 20 mM l-glutamine, and 20 ng/ml GM-CSF. After 3 d of culture at 37°C in CO2 incubator, half of the medium was replaced by fresh medium with 40 ng/ml GM-CSF. CD11c+ cDCs were purified with the Mouse CD11c Positive Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) on days 7–9 according to the manufacturer’s protocol. The purity of CD11c+ cDC was usually >90%.

**Flow cytometry**

Cells were stained in PBS containing 2% FBS at 4°C. Abs included FITC-conjugated anti-CD11c and -CD4; PE-conjugated anti-CD69; allophycocyanin-conjugated anti-CD11c, and -CD4; PE/Cy5-conjugated anti-CD80; APC-conjugated anti-CD11c and -CD4; PE-conjugated anti-CD69; allophycocyanin-conjugated anti-CD25, -CD11c, and -CD80; PE/Cy5-conjugated anti-CD86; FITC-conjugated anti-CD11b; and PE/Cy7-conjugated anti-IA/IE (MHC-II); and biotin-conjugated anti–MHC-I. Stained samples were collected using a FACSCanto II Flow Cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software version 9.0. Intracellular staining was performed using BD Cytofix/Cytoperm Kit.

**In vitro and in vivo DC-mediated T cell activation**

For in vitro T cell proliferation, 2 × 10⁵ TSC1 knockout (KO) and wild-type (WT) BM-derived DCs (BMDCs) per well/48-well plate were treated with LPS at 100 ng/ml or LPS plus OVA peptides 323–339 (OVA323-339-2 μg/ml) or OVA357–264 (SHINEKEL, 2 μg/ml) overnight. T cells were pu-
rified from OTI TCR or OTI TCR transgenic mice using the negative selection LD column (Miltenyi Biotec, Germany) and labeled with CFSE (Molecular Probes, Eugene, OR) using a previously described protocol (39). A total of 4 × 10⁵ CFSE-labeled OTI or OTII T cells were added into each well containing OVA323–339 or OVA357–264-treated WT or TSC1KO BMDCs, and then cocultured for 24 or 72 h. After 24 and 72 h, T cells were harvested and stained with the indicated Abs to assess upregulation of activation markers and proliferation, respectively, using flow cytometry. For in vivo T cell activation, 1.5 × 10⁶ CFSE-labeled Thy1.1+Thy1.2+ OTI T cells were i.v. injected into Thy1.1+ C57BL/6 mice on day 0. The recipient mice were i.v. injected with 5 × 10⁶ LPS- or LPS + OVA323–339- treated WT or TSC1KO BMDCs on day 1. Splenocytes and LN cells in the recipients were stained and analyzed on day 4.

**ELISA**

Right after 200,000 purified CD11c+ BMDCs were plated into each well of 24-well plates (Becton Dickinson Labware, Franklin Lakes, NJ), both TSC1 WT and KO BMDCs were treated with LPS at 10 ng/ml for 0, 3, 6, and 9 h. The culture supernatants were harvested to detect TNF-α, IL-12p40, and IL-6 cytokine levels determined using commercial ELISA kit (BioLegend, San Diego, CA) according to the manufacturer’s instruction. The cells were used to isolate total RNA isolation for quantitative RT-PCR (qRT-PCR).

**qRT-PCR**

The total RNA isolation, reverse transcription, and qRT-PCR were performed as previously described (31). Values for quantitative PCR (qPCR) analysis were normalized by the level of β-actin gene and are expressed as fold change over WT DCs without LPS stimulation or over MIGR1 retroviral vector–infected cells, which was arbitrarily considered as 1. The primer pairs are as follows: β-actin, forward 5’TGGCCACCTTCAGACAGATGTG-3’ and reverse 5’-AGCTGATACCCTGGTTCTAGTA-3’; TNF-α, forward 5’-CCCAAGGAGGATGAGAGTTG-3’ and reverse 5’-CAGCTTGAGTGGTTGTGCTAAGA-3’; IL-12p40, forward 5’-CTGAGGATTTGATGAAAGACAT-3’ and reverse 5’-CCAGAAGGTAATTTAATGTC-3’; IL-6, forward 5’TTCATCCAGTGTCCCTTGTG-3’ and reverse 5’TCTGGGAGTGTGATCTCTGTA-3’; IRF4, forward 5’-GAGGCTCTATTGGTGTAGACAA-3’ and reverse 5’-CCAAAGACTCACCAAGACATTG-3’; IF8, forward 5’-GATGCAAGACATGACAGAACA-3’ and reverse 5’-GCTGGTTTCAGCCTTTGCTCC-3’; MHC-I, forward 5’TGGTGGCATGACGACATTTCAA-3’ and reverse 5’-GGTTCCCAACAGCTCTAGAT-3’; MHC-II, forward 5’TGCATGCGGATCTCGAC-3’ and reverse 5’-CTCAAGACGTCGAGGTGTG-3’; TSC1, forward 5’-AGGTGTCACACCCGTGAAGA-3’ and reverse 5’-ACACGCTCCAGGTCTAAGAAGA-3’; CITA, forward 5’-CAGAGGAAGGTTTGTTTGAGA-3’ and reverse 5’-GGTCTCTTGGGTTTGTAGGC-3’; CITA promoters, mCITA-RT-PF1, forward 5’-AGTGGTCTGTCACCAAGTCT-3’, mCITA-RT-PF3, forward 5’-CTTGGCGGCCGACATC-3’, mCITA-RT-IP4, forward 5’-AGCAGGACCTCAGGAACAGC-3’, and mCITA-RT-Ex2R, forward 5’TCTGGGAGTACCTGTTAAG-3’. Western blot assay

BMDCs, seeded in six-well plates in medium containing 5 ng/ml GM-CSF overnight, were rested in serum-free medium without GM-CSF for 5–6 h and then treated with LPS (10 ng/ml) for 0, 15, 30, and 45 min. Cells were washed once in cold PBS and lysed in 1% Triton X-100, 150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA with protease and phosphatase inhibitor cocktails (Sigma). Cell lysates were subjected to immunoblotting analysis as previously described (40). Rabbit anti-TSC1, -TSC2, and -IRF8 were obtained from Cell Signaling Technology. Anti-CITA and goat anti-IRF4 Abs were from Abcam (Cambridge, MA) and Santa Cruz Biotechnology, respectively.

**Retroviral transduction**

pcDNA3-myc-CITA, pcDNA3-eFug-Reib, short hairpin RNA (shRNA) for Raptor, and shRNA control were purchased from Addgene (Cambridge, MA). Both CITA and RelB cDNA fragments were cloned into the MIGR1 retroviral vector to generate MIGR-CITA and MIGR-RelB. IRF4 was a gift from Dr. Y. Koyam (Oklahoma Medical Research Foundation). Retroviruses were generated using the Phoenix-Eco package cell line using the calcium-mediated transfection method. For infection, 2 × 10⁵ BMDCs in 1 ml cul-
ture medium on day 4 were mixed with 0.5 ml viral supernatant in a six-
well plate along with 5 μg/ml Polybrene. Cells were centrifuged at 2500 g for 1.5 h at room temperature. Medium was changed with fresh DC culture medium 7 h postinfection. Infected cells were used for experiments after 48 h postinfection. For some experiments indicated in the figures, GFP+ cells were sorted for isolating total RNA. For shRNA studies, 24 h after lentivirus infection, cells were treated with puromycin at 2 μg/ml for 4 d.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was done essentially as described previously (17, 41). In brief, BMDCs were initially treated with 1%
formaldehyde for 10 min to ensure cross-linkage followed by cell and nuclear lysis (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, 0.5 mM PMSF) and shearing. Immunoprecipitated chromatin was collected and washed sequentially with TBE buffer (20 mM Tris, pH 8.1, 50 mM NaCl, 2 mM EDTA, 0.1% SDS, 1.0% Triton X-100) and LiCl buffer (100 mM Tris, pH 8.1, 50 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA). DNA was then eluted with 50 mM NaHCO3 containing 1% SDS from the protein A/G beads (Santa Cruz) and reverse cross-linked at 65˚C overnight followed by proteinase K treatment. DNA was then purified via phenol/chloroform extraction and ethanol precipitation. For each amplification, 3 μl DNA was analyzed by real-time qPCR. The amplification primers used are mCIITA p1 forward 5’-TGG AGT CGC CTC TCA TCC A-3’; mCIITA p1 reverse 5’-TGG TGG AGT CGC CTC TCA TCA A-3’; mCIITA p3 forward, 5’-AGA GTC AGT GTT GCC TAC CA-3’; and mCIITA p3 reverse 5’-GAG TTT CAC CCA GAG TGT TG-3’.

Statistical analysis

The p values were calculated with Student t test. The p values <0.05 or <0.01 were considered significant or very significant.

Results

TSC1 is not essential for DC development

To investigate whether TSC1 plays a role in BMDC maturation, we generated TSC1 conditional KO mice carrying the ER-Cre transgene (TSC1fl/fl; TSC1KO) (31). TSC1 was efficiently deleted in BMDCs after tamoxifen treatment (Fig. 1A). Furthermore, TSC2 protein was severely decreased in TSC1KO BMDCs, which is consistent with the reported role of TSC1 on TSC2 stability in other cells (27, 31, 32). Almost all in vitro differentiated TSC1fl/fl (WT) and TSC1KO BMDCs were CD11c+ and TSC1KO BMDCs expressed similar levels of CD11c to WT BMDCs (Fig. 1B), suggesting that in vitro GM-CSF–mediated DC differentiation is not prevented by TSC1 deficiency. However, TSC1KO BMDCs expanded faster and were bigger in size than WT BMDCs during in vitro differentiation (Fig. 1C, 1D). Together, these data demonstrated that TSC1 is not essential for DC differentiation but controls DC growth and expansion in vitro.

Increased TNF-α and IL6 but decreased IL12p40 production by TSC1-deficient BMDCs

To examine how absence of TSC1 may affect TLR-mediated innate immune responses in BMDCs, we first measured TLR-induced cytokine production. After LPS stimulation, TSC1KO BMDCs produced more TNF-α and IL-6 at both the protein and mRNA levels than WT BMDCs. In contrast, IL12p40 production was reduced in TSC1KO BMDCs (Fig. 1E). To explore the mechanisms how TSC1 regulates TLR-induced cytokine production, we compared TLR signaling between WT and TSC1KO BMDCs. LPS-induced phosphorylation of IκBα, JNK1/2, and Erk1/2 was increased in TSC1KO BMDCs as compared with WT BMDCs. However, p38 phosphorylation was similar between WT and TSC1KO BMDCs (Fig. 1F).

Phosphorylation of 4E-BP1 and p70S6K, an mTORC1-dependent event, was substantially increased in TSC1KO BMDCs. However, phosphorylation of Akt at S473, an mTORC2-mediated event, was decreased in TSC1KO BMDCs (Fig. 1F). Thus, TSC1 deficiency leads to increased mTORC1 but decreased mTORC2 signaling in BMDCs. Treatment with rapamycin reduced TNF-α and IL-6 production by both WT and TSC1KO BMDCs (Fig. 1G), suggesting that mTORC1 promotes production of these cytokines. However, rapamycin exerted minimal effect on IL-12p40 production in WT BMDCs and was not able to restore IL-12 production in TSC1KO BMDCs. It has been reported that rapamycin treatment increases IL-12p40 expression (21, 22, 42–44). The inability of rapamycin to restore IL-12p40 expression in TSC1KO BMDCs suggests that TSC1 may also promote IL-12p40 expression via mTORC1-independent mechanisms.

Selective defect of MHC-II expression in TSC1KO BMDCs during maturation

An important function of DCs is to shape adaptive immunity through Ag presentation and costimulation. During DC maturation after recognition of pathogen-associated pattern molecules, DCs upregulate MHC-I and -II for effective presentation of foreign Ags to T cells, as well as CD80 and CD86, for providing costimulation to ensure full T cell activation. TSC1KO BMDCs expressed similar levels of CD80, CD86, and MHC-I compared with WT BMDCs before and after LPS stimulation. Surprisingly, cell-surface MHC-II expression on TSC1KO BMDCs was greatly decreased before and after LPS stimulation as compared with WT BMDCs (Fig. 2A). Furthermore, intracellular MHC-II but not MHC-I levels in TSC1KO BMDCs were similarly decreased, indicating an overall decrease of MHC-II expression in TSC1KO BMDCs that is not due to impaired translocation of MHC-II to the cell surface (Fig. 2B). In WT BMDCs, MHC-Ia- and β-chain mRNAs were expressed at high levels before LPS stimulation, further upregulated 3 h after LPS stimulation, and then downregulated after prolonged LPS stimulation. In TSC1KO BMDCs, both mRNAs were expressed at much lower levels than WT BMDCs. Thus, TSC1 deficiency causes decreased MHC-II expression at least at the transcriptional level (Fig. 2C). To further establish the relevance of TSC1 and MHC-II expression in DCs in vivo, we generated and analyzed TSC1fl/fl-CD11cCre mice. As shown in Fig. 2D, MHC-II but not CD80 or CD86 expression in CD11c+ DCs from TSC1fl/fl-CD11cCre mice was also reduced.

In addition to DCs, B cells and macrophages also express MHC-II and can present Ags to activate T cells. Neither TSC1-deficient B cells isolated from tamoxifen-treated TSC1fl/fl-ERCre mice nor TSC1KO BM-derived macrophages displayed decreased MHC-II mRNA or cell-surface protein expression. Moreover, IRF4 and CIITA mRNA levels in TSC1KO B cells and macrophages were not decreased as compared with WT controls (Supplemental Fig. 1). Together, these data suggest that TSC1 is selectively required for MHC-II expression in DCs.

Impaired capacity of TSC1KO BMDCs to activate CD4+ T cells

TSC1-deficient BMDCs express increased costimulatory molecules but decreased MHC-II, prompting us to explore how TSC1 deficiency may affect DC function as APCs to activate T cells. In vitro, TSC1KO DCs, pretreated with LPS plus OVA323–339 overnight, failed to induce MHC-II–restricted OVA323–339–specific CD4+ OTI T cells to upregulate CD69 or CD25, markers for early T cell activation (Fig. 3A). In contrast, WT BMDCs potently induced CD69 and CD25 upregulation in OTII T cells in an OVA323–339–dependent manner. Consistent with a defect in inducing early T cell activation, TSC1KO BMDCs loaded with OVA323–339 were unable to induce OTII T cell proliferation in a CFSE dilution assay (Fig. 3B).

To determine whether TSC1KO BMDCs are also compromised to activate T cells in vivo, we transferred CFSE-labeled Thy1.1+ Thy1.2+ OTII T cells into congenic Thy1.1 mice. Twenty-four hours later, recipient mice were immunized i.v. with WT and TSC1KO BMDCs pretreated with LPS or LPS plus OVA323–339. Seventy-two hours after DC immunization, Thy1.1+Thy1.2+ OTII T cells in the recipients immunized with WT but not TSC1KO DCs loaded with OVA (Fig. 3C) proliferated vigorously. Different from presenting Ags to CD4 T cells, the ability of TSC1KO BMDCs to present OVA257–267 (SIIFELK) to and to activate MHC-I–restricted OTII T cells was not compromised (Fig. 3D). Moreover, the decreased MHC-II expression did not occur in
TSC1f/f-ERCre BMDCs without tamoxifen treatment (Supplemental Fig. 2), further supporting that the phenotypes in TSC1KO BMDCs are dependent on deletion of TSC1. Together, these data demonstrated that TSC1 expression in DCs is critical for DCs to fulfill their function to trigger CD4 T cell activation.

Critical role of TSC1 for CIITA transcription to promote MHC-II expression in BMDCs

To explore mechanisms by which TSC1 promotes MHC-II expression in BMDCs, we first examined CIITA expression because of its essential role in MHC-II transcription (45, 46). In WT BMDCs, CIITA expression is tightly regulated and necessary for optimal MHC-II transcription. Our data show that TSC1 deficiency leads to decreased CIITA expression, which in turn results in reduced MHC-II expression.

FIGURE 1. TSC1 deficiency promotes BMDC growth and expansion. (A) Detection of TSC1 and TSC2 proteins in WT and TSC1 KO BMDCs by Western blot with indicated Abs. (B) CD11c expression on GM-CSF–induced DCs. Overlaid histograms showed CD11c staining in WT and TSC1KO BMDCs with IgG isotype as a control. (C) Enlarged cell size of TSC1KO DCs. Cell sizes of BMDCs cultured on day 7 were determined by Cellmeter Auto T4. (D) Enhanced expansion of TSC1KO DCs. Data in (C) and (D) were mean ± SD from three experiments. (E) Cytokine mRNA and protein induced in WT and TSC1KO BMDCs after LPS stimulation for the indicated times. Bar graphs indicate mean ± SD. Data are representative of four independent experiments. (F) LPS-induced signaling in WT and TSC1KO BMDCs. BMDCs were starved in serum-free medium without GM-CSF for 6 h and then treated with LPS at 10 ng/ml for 0, 15, 30, and 45 min. Cell lysates were subjected to immunoblotting analysis with indicated Abs. (G) Effects of rapamycin on LPS-induced cytokine production. WT and TSC1KO BMDCs were untreated or treated with rapamycin (20 ng/ml) 30 min before LPS stimulation for 6 h. *p < 0.05, **p < 0.01, determined by unpaired Student t test. L, LPS; R, rapamycin; Un, unstimulated.

TSC1f/f-CD11cCre BMDCs without tamoxifen treatment (Supplemental Fig. 2), further supporting that the phenotypes in TSC1KO BMDCs are dependent on deletion of TSC1. Together, these data demonstrated that TSC1 expression in DCs is critical for DCs to fulfill their function to trigger CD4 T cell activation.

FIGURE 2. Decreased MHC-II expression in TSC1KO BMDCs. In vitro differentiated WT and TSC1KO BMDCs were unstimulated or treated with LPS at 10 ng/ml overnight or for the indicated times. Cells were then used for cell-surface or intracellular staining or for making RNA. (A) Cell-surface CD80, CD86, MHC-I, and MHC-II expression on gated CD11c+ DCs. (B) Intracellular staining of MHC-I and MHC-II in gated CD11c+ DCs. (C) MHC-IIα and β mRNA levels determined by qRT-PCR. Data shown represent three experiments with the SD. (D) Decreased MHC-II expression in TSC1-deficient DCs in vivo. Overlaid histograms show MHC-II, CD80, and CD86 staining on gated CD11c+ DCs from WT and TSC1f/f-CD11cCre mice. *p < 0.05, **p < 0.01, determined by unpaired Student t test.
CIITA mRNA and protein levels were drastically decreased 3 and 6 h after LPS stimulation, respectively. In TSC1KO BMDCs, both CIITA mRNA and protein levels were substantially decreased compared with WT controls before LPS stimulation (Fig. 4A, 4B). Retroviral transduction of CIITA into BMDCs not only increased MHC-II expression in WT BMDCs, but also restored MHC-II expression in TSC1KO BMDCs to WT levels for both protein and mRNA (Fig. 4C, 4D). Together, these data suggest that decreased CIITA transcription contributes to defective MHC-II expression in TSC1KO BMDCs.

**TSC1 promotes CIITA–MHC-II expression through IRF4**

A recent study has revealed that IRF4 and IRF8 are important for MHC-II expression in splenic and BMDCs (47), and can associate with the CIITA promoter (17). However, a direct role of IRF4 in activating CIITA–MHC-II in DCs has not been demonstrated. Both IRF4 mRNA transcript and protein were severely reduced in TSC1KO BMDCs (Fig. 5A, 5B). Retroviral transduction of IRF4 into TSC1KO BMDCs considerably increased CIITA mRNA levels, as well as MHC-Ila and β mRNA levels (Fig. 5C). Moreover, IRF4 transduced (GFP+) TSC1KO BMDCs expressed much higher levels of MHC-II protein on cell surface than untransduced GFP− cells (Fig. 5D). Different from IRF4, IRF8 protein levels were not decreased in TSC1KO BMDCs (Fig. 5B). Furthermore, retroviral transduction of IRF8 into TSC1KO BMDCs failed to rescue MHC-II expression in these cells (Fig. 5E). Together, these data suggest that TSC1 promotes the expression of IRF4, which, in turn, plays a critical role for CIITA and MHC-II expression.

**IRF4 binds to CIITA promoters to control CIITA expression**

Because expression of IRF4 increased both CIITA and MHC-II expression in TSC1KO DCs, we sought to determine whether IRF4 directly activates CIITA transcription. In mouse, CIITA transcription...
is controlled by several promoters, pl, pIII, and pIV. DCs mainly use pl and, to a lesser degree, pIII (48) (Fig. 6A). Measurement of CIITA transcripts derived from these promoters by qRT-PCR showed a dramatic decrease of pl-specific CIITA transcript and a slight decrease of pIII-specific CIITA transcript in TSC1KO BMDCs compared with WT BMDCs (Fig. 6B). Using ChIP with anti-IRF4 Ab, we were able to detect association of IRF4 to both CIITA pl and pIII promoters in WT BMDCs. However, such association was reduced in TSC1KO BMDCs (Fig. 6C), which is consistent with the drastic decrease of IRF4 protein in these cells. Transduction of WT BMDCs with IRF4 resulted in 5- and 2-fold increases of pl and pIII transcripts, respectively. In TSC1KO BMDCs, both transcripts were also increased after IRF4 transduction (Fig. 6D). However, IRF4 did not increase transcription from pIV in either WT or TSC1KO BMDCs. These results, together with those in Fig. 5, demonstrate that IRF4 binds to both pl and pIII of CIITA to promote CIITA transcription, and that TSC1 regulates CIITA–MHC-II expression at least partially via maintaining IRF4 expression in DCs.

Critical role of mTORC1 signaling for CIITA–MHC-II silencing during DC maturation

As shown in Fig. 1, mTORC1 signaling was enhanced, but mTORC2 signaling was decreased in TSC1KO DCs. To determine whether enhanced mTORC1 activity leads to defective CIITA–MHC-II expression in TSC1KO BMDCs, we used shRNA to
knock down raptor, an essential component of the mTORC1 complex, in both WT and TSC1KO BMDCs. Western blot analysis showed substantial reduction of raptor protein in WT and TSC1KO BMDCs expressing shRaptor, but not a scrambled shRNA control (Fig. 7A). In WT BMDCs with shRaptor, cell-surface MHC-II expression was increased; the increase of MHC-II expression in TSC1KO BMDCs with shRaptor was much more drastic than in WT BMDCs (Fig. 7B). Compared with scrambled control shRNA, shRaptor increased IRF4 mRNA ~2-fold and CIITA mRNA ~10-fold in WT BMDCs. It also increased IRF4 and CIITA expression in TSC1KO BMDCs to levels close to WT BMDCs with the control shRNA (Fig. 7C). These observations indicate that TSC1 promotes IRF4–CIITA–MHC-II expression by inhibiting mTORC1.

TLR-induced DC maturation is accompanied by rapid silencing of CIITA and MHC-II transcription. To test whether mTORC1 plays a role in CIITA and MHC-II silencing, we treated WT BMDCs with LPS in the presence or absence of rapamycin. In immature DCs, MHC-IIα and β, as well as CIITA and IRF4, are actively transcribed. Six hours after LPS stimulation, CIITA, MHCα, and MHCβ mRNA were drastically decreased, accompanied by a modest decrease of IRF4 mRNA (Fig. 7D). Rapamycin treatment by itself did not obviously alter MHC-IIα and β mRNA levels but increased CIITA and IRF4 mRNA levels. Addition of rapamycin during LPS stimulation resulted in 2- to 3-fold increase of CIITA and IRF4 mRNA, and, importantly, MHC-IIα and β mRNA remained at high levels similar to unstimulated immature DCs (Fig. 7D). These data suggest that mTORC1 activity is critical for TLR-induced silencing of CIITA and MHC-II during DC maturation.

Discussion

After recognition of pathogen pattern molecules, immature DCs undergo an irreversible maturation process that is characterized by upregulation of cell-surface MHC-II and costimulatory molecules, and production of a variety of cytokines to induce effective and appropriate adaptive immune responses to control infection (49). Paradoxically, MHC-II and CIITA transcription is rapidly silenced during DC maturation and involves loss of histone acetylation across the gene locus and specific binding of positive regulatory domain I to the promoters (16, 17). However, the signaling mechanisms that lead to CIITA/MHC-II silencing have been unclear. In this study, we have revealed a novel function of mTORC1 in DCs for CIITA–MHC-II silencing during DC maturation and demonstrated that TSC1 is a critical regulator that controls multiple DC functions via modulating mTOR signaling.

In TSC1-deficient DCs, LPS-induced mTORC1 signaling is enhanced, but mTORC2 signaling is decreased, indicating the TSC1 differentially controls mTORC1 and mTORC2 signaling in DCs. These observations are consistent with the role of TSC1 in macrophages, as well as several other immune cell lineages such as T cells and mast cells (27–29, 31, 32). In addition to controlling mTOR signaling, TSC1 also regulates several other signaling events in DCs. TLR4-induced Erk1/2, JNK1/2, and IKK–NF-κB activation is enhanced in TSC1-deficient DCs. Because Erk1/2, JNK1/2, and IKKs are activated through different signal cascades downstream of TLR4, the data suggest that TSC1 may be involved in negative control of proximal TLR signaling. It has been reported that FoxO1 promotes TLR4 signaling, and its phosphorylation by Akt after TLR4 stimulation represents a negative feedback mechanism to prevent uncontrolled inflammatory responses (50). A potential mechanism is that the decreased mTORC2/Akt activity in TSC1KO DCs may result in elevated FoxO1 activity, leading to enhanced TLR4 signaling and activation of these downstream enzymes, as well as increased cytokine production. The ability of TSC1 to control multiple signaling events is consistent with our data showing that inhibition of mTORC1 with rapamycin is not able to fully restore normal cytokine production in TSC1-deficient BMDCs, suggesting that either mTORC2- or mTOR-independent mechanisms are involved. Further studies are needed to illustrate the mechanisms by which TSC1 regulates these canonical TLR signaling events.

One of the striking findings in our study is that TSC1/mTOR control CIITA–MHC-II expression. We have revealed that TSC1-deficient DCs express low levels of CIITA and MHC-II without obvious defects in upregulation of CD80/86 and MHC-I. The impaired CIITA–MHC-II expression in TSC1KO DCs is at least

FIGURE 7. Activated mTORC1 contributes to loss of MHC-II expression in TSC1 KO BMDCs. WT and TSC1KO BMDCs were transduced with lentivirus expressing either a control shLuc or a Raptor shRNA on day 3 followed by puromycin selection. DCs were examined on days 7 or 8. (A) Assessment of raptor protein levels by immunoblotting analysis. (B) Increased MHC-II expression in Raptor-knockdown WT and TSC1KO BMDCs. (C) Decreased Raptor protein increased CIITA and IRF4 mRNA levels in both WT and TSC1KO BMDCs determined by qRT-PCR. (D) Rapamycin treatment prevented LPS-induced CIITA/MHC-II silencing. WT BMDCs were treated with LPS or LPS plus rapamycin for 6 h. MHC-IIα, MHC-IIβ, CIITA, and IRF4 mRNA transcripts from treated cells were determined by qRT-PCR. Data are representative of three independent experiments. *p < 0.05, determined by unpaired Student t test.
partially caused by enhanced mTORC1 signaling in these cells because knocking down raptor expression in TSC1KO DCs almost restores CIITA–MHC-II expression to WT levels. The inhibitory effects of mTORC1 for CIITA–MHC-II expression can also be observed in WT DCs as raptor knockdown in these cells upregulates CIITA–MHC-II expression. Furthermore, acute treatment of WT DCs with rapamycin prevents TLR4-induced CIITA–MHC-II silencing during DC maturation. Because rapamycin treatment only slightly increased IRF4 expression, this suggests that mTORC1 may also suppress CIITA–MHC-II expression through IRF4-independent mechanisms or that rapamycin-resistant functions of mTORC1 may regulate IRF4 expression in DCs. Of note, Erk1/2 have been previously found to be involved in CIITA–MHC-II silencing (51). In addition, Erk1/2 promotes mTOR signaling in T cells (40). The elevated Erk1/2 activity in TSC1KO DCs may also contribute to downregulation of MHC-II expression in these cells.

CIITA expression is controlled by different promoters in different cell lineages. In cDCs and macrophages, CIITA is transcribed primarily from pI and to a lesser extent from pII. In B cells and pDCs, CIITA is mainly transcribed from pII and is critical. pIV is primarily from pI and to a lesser extent from pIII. In B cells and other cell lineages. In cDCs and macrophages, CIITA is transcribed to contribute to downregulation of MHC-II expression in these cells.

Developmental in-depth understanding of mTOR signaling in different cell lineages is needed before manipulating mTOR signaling in the innate immune cells for treatment of immune-related diseases. On one hand, it inhibits the production of certain inflammatory cytokines such as IL-6 and IFN-α, and the upregulation of costimulatory molecules. On the other hand, it promotes IL-12 production and is essential for activation of IRF4-CIITA-MHCII transcription. Our data also reveal that mTORC1 is critical for CIITA–MHC-II silencing during DC maturation, and that TSC1 modulates DC function at least partially by inhibiting mTORC1 function.

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Disclosures
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References


