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mTORC2 controls Th9 polarization and allergic airway inflammation

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Abstract

Background: T helper type 9 (Th9) cells, a subpopulation of $CD4^+$ T cells, play a critical role in the pathogenesis of allergic airway inflammation. However, it remains unknown whether mTORC2 regulates Th9 differentiation or function during allergic inflammation.

Methods: T-cell-specific Rictor-deficient mice, a mouse model of allergic airway inflammation induced by ovalbumin (OVA) sensitization and a mouse model of adoptive transfer of induced Th9 cells, were used to address the roles of mTORC2 in the pathogenesis of allergic airway inflammation. The in vitro Th9 induction, multiple colors flow cytometry, real-time PCR, and Western blots were used to investigate the molecular effects of mTORC2 in Th9 induction.

Results: The differentiation of naïve $CD4^+$ T cells into Th9 cells was significantly diminished in the absence of Rictor, the core component of mTORC2. Using a mouse model of allergic airway inflammation induced by OVA sensitization, T-cell-specific Rictor-deficient mice show much less severe allergic airway inflammation characterized by decreased pathological alterations and fibrosis of the lungs, which was accompanied with reduced Th9 differentiation and infiltration. Importantly, the isolated Rictor-deficient Th9 cells mediate less severe allergic pathogenesis upon adoptive transfer. Rictor deficiency impairs Th9 cell differentiation by reducing IRF4 expression rather than affecting Foxo1/Foxo3a transcriptional activity, which is likely due to decreased Akt and/or STAT6 activation.

Conclusions: These findings uncover a novel role of mTORC2 in Th9 cell differentiation and may have important implications for therapeutic intervention of allergic diseases.

KEYWORDS
allergy, asthma, mTOR, Rictor, Th9 cells

Abbreviations: AP-1, activator protein 1; BAL, bronchoalveolar lavage; dLNs, draining lymph nodes; CFSE, carboxyfluorescin diacetate succinimidyl ester; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; GATA3, GATA binding protein 3; H&E, hematoxylin and eosin; ILC2s, type 2 innate lymphoid cells; IRF4, interferon regulatory factor 4; iTreg, induced regulatory T cells; MAPK, mitogen-activated protein kinases; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; OVA, ovalbumin; PAS, periodic acid-Schiff reagent; PBS, phosphate-buffered saline; STAT, signal transducer and activator of transcription; Th, T helper; WT, wild type

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1 | INTRODUCTION

Allergic asthma is one of the most common chronic diseases resulting in a substantial burden of diseases in the world. Although rates of deaths caused by asthma worldwide have reduced greatly over the past decades, no available therapeutic regimens can cure asthma until now. Current clinical therapies based on inhaled corticosteroids and long-acting β2 agonists are effective in controlling allergic asthma in most, but not all patients.[1] Allergic asthma is a chronic inflammatory disease of the airways characterized by infiltration of eosinophils, basophils, mast cells, and CD4 T helper (Th) cells into the airway submucosa. Th2 cells and type 2 innate lymphoid cells (ILC2s) producing IL-4, IL-5, and IL-13 are now recognized as important cells underlying allergic eosinophilic asthma.[2,3] The innate immune cytokines such as IL-1, IL-25 and IL-33, and thymic stromal lymphopoietin released from airway epithelial cells can activate Th2 cells and ILC2s.[3,4] In addition, IL-9 expression is significantly higher in lungs of asthmatic patients.[5,6] Transgenic expression of IL-9 results in allergic inflammation.[7-9] IL-9 promotes mucus production from lung epithelial cells and pulmonary mastocytosis.[10] Using a conventional acute allergic airway inflammation mouse model with ovalbumin (OVA)/alum immunization followed by aerosolized airway OVA challenges, a significant decrease in airway hyper-responsiveness and bronchoalveolar lavage (BAL) eosinophilia after administration of anti-IL-9 blocking antibody occurs.[11] A recent study by Chang et al.[12] showed that Th9 cells were involved in the lung allergic inflammation. Adoptively transferred Th9 cells lead to allergic airway disease characterized by increased amounts of eosinophil recruitment, tissue mast cell numbers, and serum IgE following OVA challenge,[13-15] supporting the contribution of Th9 cells to allergic inflammation.

Upon TCR stimulation, naive $CD4^+$ Th cells are activated and differentiate into one of the distinctive subpopulations including Th1, Th2, Th9, Th17 cells, or induced regulatory $CD4^+CD25^+$ T cells (iTreg), depending on the cytokine milieu.[16,17] IL-9-producing $CD4^+$ Th cells (Th9 cells) are induced from naive $CD4^+$ T cells by transforming growth factor-β (TGF-β) and IL-4 during T-cell activation. Multiple transcription factors have been shown to regulate Th9 differentiation. For instance, IL-4 activates signal transducer and activator of transcription (STAT) 6 and interferon regulatory factor 4 (IRF4), whereas TGF-β activates PU.1 and represses T-box expressed in T cells (T-bet) and GATA binding protein 3 (GATA3), and the integration of those events eventually drives IL-9 expression.[18] Emerging evidence supports the fact that there are potential binding sites at IL-9 promoter or enhancer region for transcription factors including PU.1, IRF4, STATs, nuclear factor of activated T cells, GATA1, GATA3, Smads, and Notch as well as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1).[12,18,19] However, the intracellular molecular regulatory network on Th9 differentiation remains largely unknown so far.

The kinase mTOR is an evolutionarily conserved member of the phosphatidylinositol-3-OH kinase (PI3K)-related kinase family that plays a central regulatory role in cellular metabolism, protein synthesis, energy balance, proliferation, and survival.[20] In general, mTOR forms two functionally distinct multiprotein complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Raptor and Rictor are the core components of mTORC1 or mTORC2, respectively.[21] Deletion of Raptor or Rictor leads to the loss of mTORC1 or mTORC2 activity, respectively.[22] The mTORC1 complex promotes phosphorylation of the translational regulators S6K1 and 4E-BP1 and is believed to have a central role in regulating cellular growth, proliferation, and metabolism. mTORC1 has been well shown to regulate both innate and adaptive immunity.[23-32] Indeed, mTORC1 is required for Th1, Th2, Tfh, Th17, and iNKT cell differentiation.[25,29,33,34] The activity of mTORC2 can be measured by phosphorylation of the hydrophobic motif of Akt at Ser473, serum- and glucocorticoid-regulated kinase (SGK1), and Protein kinase C alpha (PKC-α).[35] Recently, the Rictor containing mTORC2 or its downstream target SGK1 were shown to promote Th2 differentiation,[36-38] and mTORC2 was also involved in regulating CD8 effector or memory differentiation.[23,24] However, the role of mTORC2 in $CD4^+$ T-cell polarization into Th9 subset is still unknown.

In this study, we employed T-cell-specific Rictor-deficient mice to address the role of mTORC2 in Th9 differentiation. We found that Rictor deficiency significantly impaired Th9 cell differentiation through regulating Akt/IRF4 signaling axis. Importantly, T-cell-specific Rictor-deficient mice show significantly decreased allergic response in an OVA-induced allergic airway inflammation mouse model. Thus, our study revealed a novel function of mTORC2 in Th9 cell development and identified the mTORC2/Akt signaling axis as a potential target for therapeutic intervention of allergic airway inflammation in clinics.

2 | MATERIALS AND METHODS

2.1 | Mice

$Rictor^{loxp/loxp}$ mice were kindly provided by Dr. Zhongzhou Yang from Center of Model Animal Research at Nanjing University, China. Foxo3a KO mice were originally generated and kindly provided by Dr. Lianfeng Zhang from Chinese Academy of Medical Sciences & Comparative Medical Center, Peking Union Medical College, China. C57BL/6 (B6) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). Lck-cre, and OT II transgenic mice and the other mice were bred and maintained under specific pathogen-free conditions in the animal facility of Institute of Zoology, Chinese Academy of Science. Sex-matched littermate mice of 8-12 weeks were mainly used for experiments. All animal studies were approved by the Animal Ethics Committee of the Institute of Zoology (Beijing, China).

2.2 | Differentiation of $CD4^+$ T-cell subpopulations from naïve T cells in vitro

Naïve T cells ($CD4^+CD44^-CD62L^+$) were purified from spleens of wild type (WT) or RictorKO mice by MoFlo XDP (Beckman Coulter, Brea, CA, USA) or magnetic cell sorting (Stem cell). Naive $CD4^+$ T cells (1×10^6 cells/mL in 96-well plates) were activated with plate-bound anti-CD3 (2 μg/mL; 145-2C11; R&D Systems, Minneapolis, MN, USA) and soluble anti-CD28 (2 μg/mL; 37.51; BD Biosciences, San Jose, CA, USA) and cultured under Th9 induction conditions (IL-4: 20 ng/mL; R&D Systems; TGF-β: 2 ng/mL; R&D Systems; and

anti-IFN-γ: 10 μg/mL, XMG1.2; eBioscience, San Diego, CA, USA); Th2 induction conditions (IL-4: 20 ng/mL; R&D Systems; and anti-IFN-γ: 10 μg/mL, XMG1.2; eBioscience, San Diego, CA, USA); Th1 induction conditions (IL-12: 10 ng/mL; PeproTech, Rocky Hill, NJ, USA; IL-2: 100 U/mL, PeproTech; and anti-IL-4: 10 μg/mL, 11B11; eBioscience); Th17 induction conditions (TGF-β: 1 ng/mL; R&D Systems; IL-6: 50 ng/mL; R&D Systems; anti-IFN-γ: 10 μg/mL, XMG1.2; eBioscience; anti-IL-4: 10 μg/mL, 11B11, eBioscience; and anti-IL-2: 10 μg/mL, JES6-1A12; eBioscience); and Treg induction conditions (TGF-β: 1 ng/mL; R&D Systems; IL-2: 100 U/mL; PeproTech). After 3 days or the indicated time points of differentiation, cell polarization was assessed by intracellular staining and real-time PCR.

2.3 | Flow cytometry

After 3-5 days of culture, cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) for 5 hours at 37°C and Golgistop (BD Pharmingen, San Diego, CA, USA) was added to the cells for the last 3 hours of stimulation. Intracellular cytokine staining was carried out with Fixation/Permeabilization buffer solution according to the manufacturer's protocol (BD Bioscience). Fluorochrome-conjugated anti-mouse IL-9 (RM9A4; BioLegend, San Diego, CA, USA), anti-mouse IL-4 (11B11; eBioscience), anti-mouse IL-17A (TC11; BioLegend), anti-mouse IFN-γ (XMG1.2; BioLegend), and anti-mouse Foxp3 (NRRF-30; eBioscience) were used for staining. Foxp3 staining was carried out with Fixation/Permeabilization buffer solution according to the manufacturer's protocol (eBioscience). Stained cells were analyzed on a FACSCalibur (BD Bioscience) or Epics XL (Beckman Coulter) with FCS Express 3 software (De Novo Software, Los Angeles, CA, USA).

2.4 | Real-time PCR

Total RNA was extracted from cultured cells with a total RNA kit (Promega, Madison, WI, USA), and cDNA was synthesized with cDNA Reverse Transcription kit (Takara, Kusatsu, Shiga, Japan). Quantitative real-time PCR was performed on the CFX96 real-time system (Bio-Rad, Hercules, CA, USA) using Power SYBR® PCR Master Mix (Applied Biosystems, Foster City, CA, USA). All real-time PCRs were carried out using optical 96-well reaction plate and run in triplicate.[39] Each plate included two no template controls. The primers used in this study are summarized in Table 1. The real-time PCR data were analyzed using comparative C_t method and were normalized using the housekeeping gene HPRT.

2.5 | ELISA

IL-9 in cell culture supernatants and homogenate of lung tissues was measured with ELISA kit according to the manufacturer's instructions (eBioscience).

2.6 | Induction of allergic airway inflammation

Allergic airway inflammation was induced as described.[26] Mice of 8-10 weeks were sensitized by intraperitoneal injection on days 0 and 14 of OVA (Sigma-Aldrich) adsorbed with aluminum hydroxide (Sigma-Aldrich) at a dose of 10 μg OVA per 1 mg aluminum hydroxide. From day 21, mice were exposed to aerosolized OVA (1% w/v) for 30 minutes per day for 5-6 consecutive days. Mice were killed 48 hours after the final intranasal challenge.

The trachea was cannulated, and lungs were lavaged with 3 mL PBS. Cell numbers in BAL fluid were counted with a hemocytometer. Cells in BAL fluid were analyzed by flow cytometry with antibodies of CD11b (M1/70; eBioscience), Siglec F (E50-2440; BD Pharmingen), Ly6G (1-A8; BD Biosciences), CD3e (145-2C11; eBioscience), B220 (RA3-6B2; Biolegend), and F4/80 (BM8; eBioscience) analyzed. RNA was isolated from cells in BAL fluid for real-time PCR analysis. Peripheral immune responses in sensitized and challenged mice were assessed by culture of splenocytes for 72 hours with OVA (100 μg/mL). Lung tissue samples were prepared from control mice and mice in OVA-induced allergy inflammation, embedded in paraffin, and sectioned, then stained with hematoxylin and eosin (H&E) and periodic acid-Schiff reagent (PAS) for evaluation of the infiltration of inflammatory cells and mucin production in trachea.

2.7 | Immunoblot

Naïve $CD4^+$ T cells were cultured in 1640 medium with 10% fetal bovine serum and essential supplements under certain polarization settings. After stimulation, cells were washed once in cold PBS and lysed in RIPA buffer (50 mmol/L Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA pH 7.4) with protease and phosphatase inhibitor cocktails (Roche) for 10 minutes on ice. Protein concentration was determined using a BCA assay.[40] The lysates were mix with 5× SDS-PAGE loading buffer and boiled at 100°C for 5 minutes. Protein samples were size-fractionated on 10% SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After blocked for 1 hour, then the membranes were incubated with primary antibodies overnight at 4°C. The appropriate HRP-conjuncted secondary antibody was then added and was detected through chemiluminescence (Millipore). Actin was used as a protein loading control. Phospho-STAT6 (Tyr641), STAT6, phospho-Smad3 (Ser423/425), Smad3, phospho-AKT (Ser473), AKT, phospho-NDRG1 (Thr346), NDRG1, phospho-FoxO1 (Thr24)/FoxO3a (Thr32), FoxO1, RelB, β-Actin, and histone H3 were purchased from CST company.

2.8 | Statistical analysis

All data were presented as mean±SD. Unpaired two-tailed Student's *t*-test was used for data of two groups. Data more than two groups were subjected to one-way analysis of variance (ANOVA) with SAS 9.2 version (SAS Institute Inc, Cary, NC, USA). Comparisons were considered statistically significant when the *P* value was <.05.

3 | RESULTS

3.1 | Rictor is selectively required for Th9 cell differentiation

Recently, Th9 cells are well defined as a distinct T helper cell lineage by secreting large amounts of IL-9 upon stimulation and promote allergic asthma pathogenesis. Given decreased Th2 differentiation in the absence of mTORC2, we wanted to examine whether loss of mTORC2 activity affected Th9 differentiation. To this end, we have generated mice with specific loss of mTORC2 activity in T cells by deleting the core mTORC2 component Rictor with Lck-Cre mice (Rictor KO, Rictor$^{loxp/loxp}$:Lck-Cre^{+}). We first confirmed the successful deletion of Rictor at both mRNA and protein level in CD4^{+} T cells of Rictor KO mice (Fig. S1). Next, naïve Rictor-deficient and WT (Rictor$^{fl/fl}$; Lck-cre-) CD4^{+} T cells were activated with anti-CD3/anti-CD28 mAbs in the presence of TGF-β and IL-4 to induce Th9 polarization. WT CD4^{+} T cells expressed high level of IL-9 mRNA upon TCR activation as detected by real-time PCR (Figure 1A), as reported previously.[41] However, Rictor-deficient CD4^{+} T cells under the same polarization settings show remarkably less IL-9 mRNA expression as compared with WT CD4^{+} T cells in all detected time points ($P<.001$, Figure 1A), implying that the decreased IL-9 expression at the transcription level in the absence of mTORC2 is not due to the altered response kinetics. The decreased IL-9 expression in Rictor-deficient CD4^{+} T cells was further confirmed by the reduced concentrations of IL-9 in the culture medium as indicated by ELISA ($P<.01$, Figure 1B). Furthermore, we reproducibly observed the lower expression of IL-9 mRNA expression in Rictor-deficient CD4^{+} T cells in all culture systems with different concentrations of TGF-β and IL-4, respectively ($P<.001$, Figure 1C,D). Consistently, the frequency of IL-9^{+} T cells in Rictor-deficient CD4^{+} T cells was significantly decreased in comparison with WT cells as determined by flow cytometric analysis ($P<.01$, Figure 1E). When sorted Rictor-deficient and WT naïve CD4^{+} T cells were stimulated with anti-CD3/anti-CD28 mAbs, they showed comparable upregulation of the activation markers CD25 and CD69 ($P>.05$, Fig. S2A,B). In line with those observations, naïve Rictor-deficient CD4^{+} T cells display similar levels of proliferation and survival as compared to WT cells upon activation as determined by carboxyfluorescin diacetate succinimidyl ester (CFSE) dilution assays ($P>.05$, Fig. S2C) and propidium iodide staining ($P>.05$, Fig. S2D), respectively. Thus, all these observations argue against the fact that the decreased IL-9 production in the absence of Rictor was due to defective T-cell activation and proliferation.

As expected, Rictor deficiency significantly decreased Th2 cell differentiation ($P<.01$, Fig. S3A-C), which is consistent with the previous reports.[37] However, either the differentiation toward Th1 or Th17 was not affected with Rictor deficiency, albeit slightly but significantly reduced differentiation into Foxp3 expressing regulatory T cells occurs ($P>.05$, Fig. S3A-C). Consistently, we observed that the key transcript factors T-bet and RORγt for Th1 and Th17, respectively, were expressed at similar level, whereas the expression of Th2 specification factor GATA3 was reduced in the absence of

TABLE 1 Primers used for genotyping and real-time PCR

Gene name	Sequence (5′→3′)	Usage
Lck-cre	TGGGAGGCAGGAAGTGGGTAACTAGACTAA	Genotyping
	GAAGATAATCGCGAACATCTTCAGG	
	CTCTTCCGTGATGTATGCTA	
Rictor	ACTGAATATGTTCATGGTTGTG	
	GAAGTTATTCAGATGGCCCAGC	
Foxo3a	ATTCCTTTGGAAATCAACAAAACT	
	TGCTTTGATACTATTCCACAAACCC	
	AGATTTATGTTCCCACTTGCTTCCT	
IL-9	F: CTCTTGCCTGTTTTCCATCG	RT-PCR
	R: CCCAGGAGACTCTTCAGAAATG	
IFN-γ	F: GAACTGGCAAAAGGATGGTGA	RT-PCR
	R: TGTGGGTTGTTGACCTCAAAC	
IL-4	F: CGAATGTACCAGGAGCCATATC	RT-PCR
	R: TCTCTGTGGTGTTCTTCGTTG	
IL-17A	F: CTCAGACTACCTCAACCGTTCC	RT-PCR
	R: ATGTGGTGGTCCAGCTTTCC	
FoxP3	F: ACCATTGGTTTACTCGCATGT	RT-PCR
	R: TCCACTCGCACAAAGCACTT	
T-box21	F: AGCAAGGACGGCGAATGTT	RT-PCR
	R: GGGTGGACATATAAGCGGTTC	
GATA3	F: AGGGACATCCTGCGCGAACTGT	RT-PCR
	R: CATCTTCCGGTTTCGGGTCTGG	
Rictor	F: GAGAAAGCTGGGCCATCTGAAT	RT-PCR
	R: GCAACCCGGCTGCTCTTACTT	
RORC	F: GACCCACACCTCACAAATTGA	RT-PCR
	R: AGTAGGCCACATTACACTGCT	
IL-5	F:AGATTCCCATGAGCACAGTG	RT-PCR
	R: TGTCTAGCCCCTGAAAGATTTC	
IL-13	F: GCATGGTATGGAGTGTGGAC	RT-PCR
	R: ATTGGAGATGTTGGTCAGGG	
IL-2	F: CCTGAGCAGGATGGAGAATTACA	RT-PCR
	R: TCCAGAACATGCCGCAGAG	
IL-21	F: CCAAACTCAAGCCATCAAACC	RT-PCR
	R: CTCATACGAATCACAGGAAGGG	
IRF4	F: TCCTCTGGATGGCTCCAGATGG	RT-PCR
	R: CACCAAAGCACAGAGTCACCTG	
BCL6	F: CATACAAATGTGATCGCTGCC	RT-PCR
	R: GAGTGTGGGTCTTCAGGTTG	
BATF	F: GCAGTGACTCCAGCTTCAG	RT-PCR
	R: TGTCGGCTTTCTGTGTCTG	
Muc5ac	F: AAAGACATAGTCACTCAGCAA	RT-PCR
	R: CTGGGAAGTTCAGTGTCAAACCA	
HPRT	F: AGTACAGCCCCAAAATGGTTAAG	RT-PCR
	R: CTTAGGCTTTGTATTTGGCTTTTC	

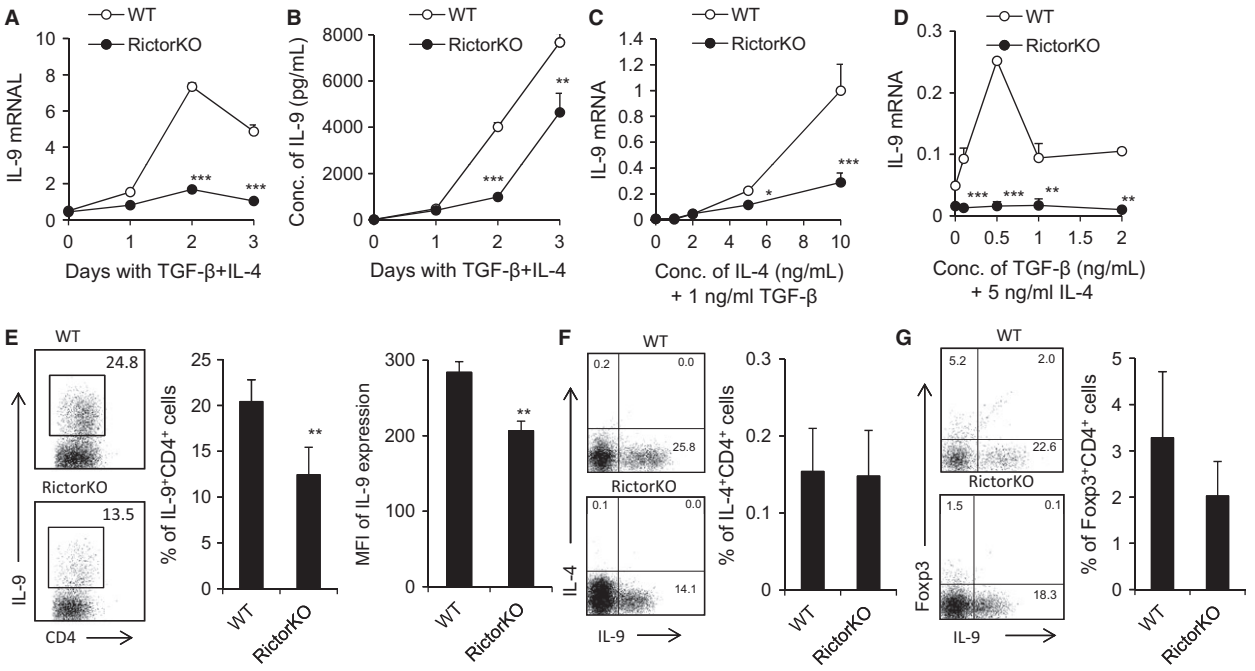

FIGURE 1 Rictor deficiency selectively impairs Th9 differentiation. (A) IL-9 mRNA expression of naïve WT and RictorKO CD4$^+$ T cells cultured under Th9-skewing condition for different periods (0-3 days). (B) ELISA analysis to measure IL-9 level in the culture medium of WT and RictorKO naïve CD4$^+$ T cells cultured under Th9-skewing condition. IL-9 mRNA expression after naïve WT and Rictor KO CD4$^+$ T cells cultured under Th9-skewing condition with different doses of IL-4 (C) or TGF-β (D) for 48 hours as indicated. (E) Representative FACS plots showing intracellular staining of IL-9 in CD4$^+$ T cells differentiated for 72 hours with anti-CD3 and anti-CD28 mAbs plus TGF-β and IL-4. The frequency of CD4$^+$IL-9$^+$ cells was summarized (right). Representative FACS plots to show intracellular staining of IL-9/IL-4 (F) or IL-9/Foxp3 (G) in CD4$^+$ T cells cultured under Th9-skewing condition for 72 hours. Data are shown as mean±SD (n=3), representing one of three independent experiments. *P<.05, **P<.01, and ***P<.001 compared with WT cells or the controls

functional mTORC2 at the mRNA level (Fig. S4). On the other hand, lower but similar levels of IL-4$^+$ T cells and Foxp3$^+$ T cells were detected in WT and Rictor-deficient T cells cultured under Th9-inducing system (Figure 1F,G), indicating that the poor Th9 differentiation of Rictor-deficient CD4$^+$ T cells is not due to the skewed differentiation into either IL-4-induced Th2 cells or TGF-β-induced Foxp3$^+$ T cells. Altogether, our findings suggest that mTORC2 is selectively required for Th9 and Th2 differentiation.

3.2 | mTORC2 controls Th9 cell differentiation in a Foxo1/Foxo3a-independent manner

It is reported that mTORC1 and mTORC2 do not regulate cell surface IL-4R (CD124) expression on T cells,[37] which was also confirmed in our study by real-time PCR and flow cytometric assay (Fig. S5). To understand the mechanisms behind dysregulated Th9 cell differentiation with Rictor deficiency, we first examined the expression pattern of several known regulators important for Th9 expression in WT and Rictor-deficient T cells by Western blots and real-time PCR. As expected, we noticed a dramatic decrease in Akt (p-S473) phosphorylation in Rictor KO CD4$^+$ T cells, which confirmed the functional mTORC2 deficiency by deletion of Rictor in T cells (Figure 2A). Meanwhile, phosphorylation of N-myc downstream-regulated gene 1 (NDRG1), another downstream target of mTORC2, is also greatly diminished in the absence of Rictor (Figure 2A). As reported previously, the reduced Akt activity in the absence of mTORC2 leads to decreased phosphorylation of Foxo1/Foxo3a, which then drives their nuclear accumulation. Indeed, we observed that Foxo1 was mostly localized to the nuclei of Rictor KO cells, whereas it was mainly cytoplasmic in WT CD4$^+$ T cells (Figure 2B). To further explore the role of Foxo1 or Foxo3a in regulating Th9 differentiation, we knocked down Foxo1 with short hairpin RNA (shRNA) and asked whether reduction in Foxo1 rescue the decreased IL-9 production. Surprisingly, we found that inhibition of Foxo1 suppressed IL-9 production and Th9 differentiation (Figure 2C,D). Furthermore, FoxO3aKO naïve CD4$^+$ T cells showed decreased ability to differentiate into Th9 cells in vitro (Figure 2E,F). Therefore, our data suggest that the increased Foxo1/FoxO3a activity in RictorKO T cells is not responsible for the poor Th9 induction.

3.3 | mTORC2 controls Th9 cell differentiation via regulating IRF4 downstream of either Akt or STAT6

Next, we further explored the downstream effectors of either IL-4 or TGF-β signaling pathway to understand the impaired Th9 differentiation with Rictor deficiency. Indeed, we observed the upregulation of phosphorylated STAT6 in WT CD4$^+$ T cells under Th9 polarization. However, phosphorylation of STAT6 was markedly reduced in Rictor-deficient T cells (Figure 3A), which is consistent with previous findings demonstrating that Rictor-deficient Th2 cells

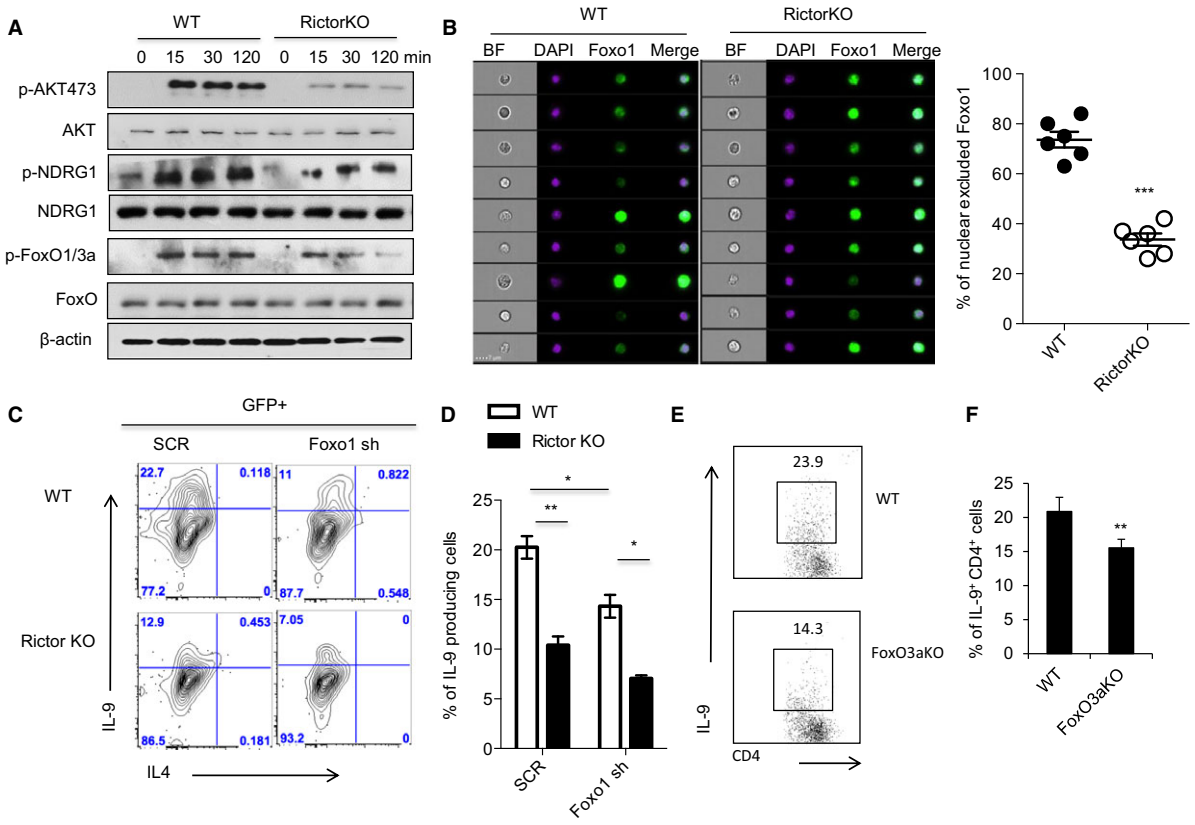

FIGURE 2 mTORC2 controls Th9 cell differentiation in a Foxo1/Foxo3a independent manner. (A) Immunoblot analysis of p-Akt473, p-Foxo1/Foxo3a, p-NDRG1 and total Akt, FoxO1 and NDRG1 in naïve T cells from WT and RictorKO mice polarized under Th9 conditions for the indicated time points (0-120 minutes). (B) Imagestream analysis of Foxo1 localization in WT or Rictor KO $CD4^+$ T cells under Th9 polarization. (C) Representative FACS plots showing IL-9 expression with either SCR or Foxo1 shRNA in WT or Rictor KO $CD4^+$ T cells during Th9 polarization (D) and the frequencies of IL-9^+ T cells are summarized (E) Representative FACS plots showing intracellular staining of IL-9 in WT and FoxO3aKO $CD4^+$ T cells differentiated for 72 hours under Th9 conditions. (F) The frequency of $CD4^+IL\text{-}9^+$ cells in WT and FoxO3aKO $CD4^+$ T cells differentiated for 72 hours under Th9-skewing condition was summarized. Data are shown as mean±SD (n=3), representing one of three independent experiments. *P<.05, **P<.01 and ***P<.001 compared with WT cells or the controls. [Colour figure can be viewed at wileyonlinelibrary.com]

show impaired STAT6 phophorylation.[37] Moreover, the phosphorylation of Smad3 downstream of TGF-β signaling was also decreased in the absence of mTORC2, whereas the phosphorylation of STAT5 in $CD4^+$ T cells was unaffected by Rictor deficiency (Figure 3A).

Interferon regulatory factor 4 (IRF4) is essential for Th9, as well as Th2 and Th17 differentiation. Consistent with the decreased IL-9 induction, we found IRF4 mRNA expression was reduced in IL-4-stimulated Rictor-deficient cells under Th9 polarization, indicating diminished *Irf4* transcription in the absence of mTORC2 (Figure 3B). We also confirmed the decreased IRF4 expression at the protein level (Figure 3C). Altogether, our data suggest that mTORC2 supports IRF4 expression in Th9 cells stimulated with IL-4. To further prove the function of Akt activity downstream of mTORC2 in regulating IRF4 expression, and thus IL-9 production, we included the inhibitors for PI3K or Akt during Th9 induction. Indeed, inhibition of either PI3K with LY294002 (Fig. S6) or Akt with 124005 (Figure 3D) significantly reduces the expression of IRF4. Interestingly, the decreased IRF4 expression in the presence of PI3K (Fig. S6) or Akt inhibitor could be extended to less IL-9 production as evidenced by intracellular staining with flow cytometry (Figure 3E,F) and real-time PCR (Figure 3G).

Previous studies with sequence and bioinformatics analysis have identified the potential binding sites in *Il-9* promoter region for multiple transcription factors.[18,19] It is shown that OX40 is highly selective and potent in Th9 induction via noncanonical NF-kB pathway. However, we observed similar phosphorylation and localization into the nuclei of RelB between WT and Rictor-deficient $CD4^+$ T cells under Th9 induction (Fig. S7). IL-4-activated STAT6 is required for repression of T-bet and Foxp3 expression in Th9 cells. Meanwhile, STAT6 is also required for IRF4 induction, which thus promotes Th9 differentiation. In the absence of STAT6, there is decreased IRF4 expression as well as reduced binding of IRF4 to IL-9 gene. Therefore, STAT6-deficient T cells fail to produce IL-9. Given the fact that both Akt and STAT6 activation are impaired in the absence of mTORC2 under Th9 polarization conditions, which could contribute to downregulated IRF4 activity, and we conclude that Rictor regulates Th9 cell differentiation by modulating IRF4 expression or activity through STAT6 and Akt signaling pathways.

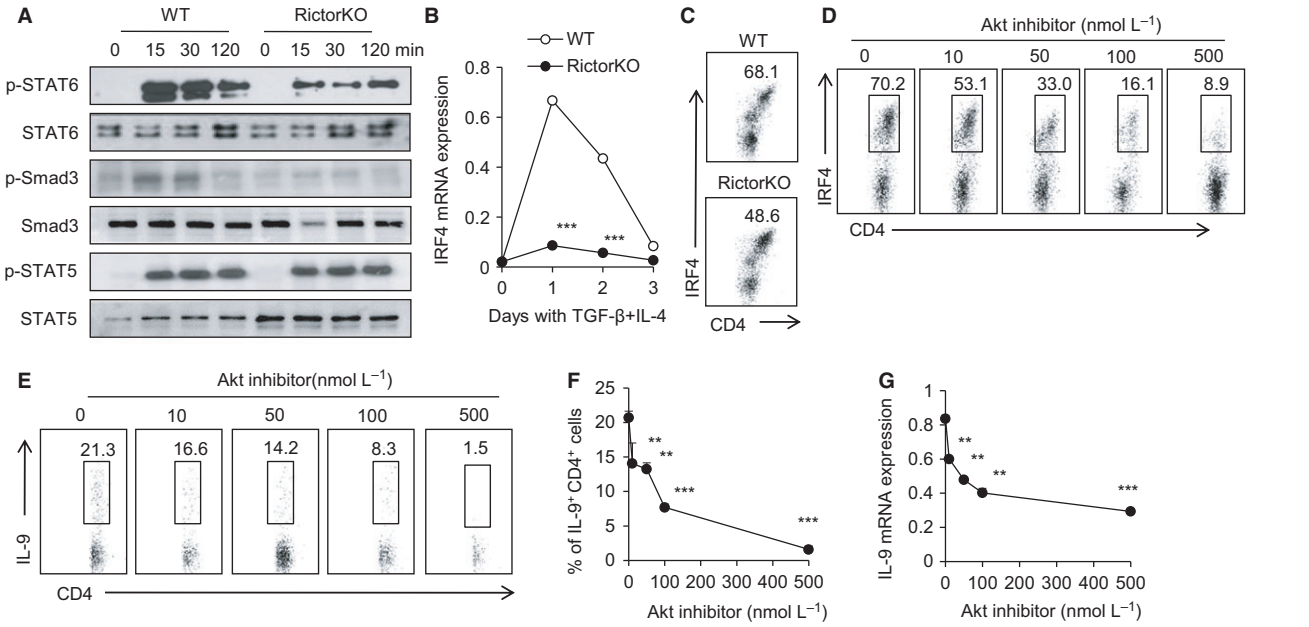

FIGURE 3 mTORC2 controls Th9 cell differentiation via regulating IRF4 downstream of either Akt or STAT6. (A) Immunoblot analysis of p-STAT6, p-Smad3, p-STAT5 and total STAT6, Smad3 and STAT5 in naïve T cells from WT and RictorKO mice polarized under Th9 conditions for the indicated time points (0-120 minutes). (B) IRF4 mRNA expression of naïve WT and Rictor KO $CD4^+$ T cells cultured under Th9-skewing condition for different periods (0-3 days). (C) Intracellular staining of IRF4 in $CD4^+$ T cells differentiated for 72 hours under Th9-skewing condition. (D) The percentage of IRF4 expression in $CD4^+$ T cells differentiated for 72 hours under Th9-skewing condition in the presence of different doses of Akt inhibitor. Intracellular staining of IL-9 in $CD4^+$ T cells (E) and the frequency of $IL\text{-}9^+$ T cells (F) or IL-9 mRNA expression (G) in the presence of Akt inhibitor were shown. Data are shown as mean±SD (n=3), representing one of three independent experiments. *P<.05, **P<.01, and ***P<.001

3.4 | T-cell-specific RictorKO mice are resistant to allergic airway inflammation

To further prove the function of mTORC2 in Th9 differentiation, we employed an OVA-induced allergic airway inflammation mouse model, in which Th9 cells play a critical role as reported recently.[42] Both OVA-presensitized RictorKO and WT littermates were thus challenged with aerosolized OVA. Indeed, we observed significantly higher OVA-specific IgE levels in the sera of WT littermates upon allergic induction by OVA as determined by ELISA assays (P<.01, Figure 4A), which is consistent with previous reports.[42] However, there was only a slight upregulation of OVA-specific IgE in the sera of RictorKO mice after similar OVA induction (P<.001, Figure 4A), indicating less strong allergic response by specific deletion of mTORC2 in T cells. Furthermore, hematoxylin and eosin (H&E) staining confirmed that RictorKO mice suffered less severe pathogenesis of allergic airway inflammation in the lung tissues than their WT counterparts as indicated by the reduced pathological changes (Figure 4B). The reduced severity of OVA-induced allergic airway inflammation in RictorKO mice was also supported by a significant less infiltration by leukocytes and eosinophils in the BAL fluid of RictorKO mice than those in WT mice after OVA challenge (P<.01, Figure 4D,E). Mucus production in bronchus of lung tissues can reflect the severity of allergic inflammation and can be evaluated by Periodic acid Schiff reagent (PAS) staining.[43] RictorKO mice harbored less mucus in lung tissues (as indicated by arrows) compared with WT mice in OVA-induced allergic airway inflammation mouse model (Figure 4F). The higher expression of *Muc5ac* and *Mcpt1* upon OVA challenge reflects mucus metaplasia and mast cell proteases, respectively.[44] Consistent with the less severity of allergic response, both *Muc5ac* expression and *Mcpt1* expression at the mRNA level in the cells of BAL fluid of allergic airway inflammation-induced RictorKO mice were significantly lower than those in allergic airway inflammation-induced WT controls (P<.01, Figure 4G). Importantly, the IL-9 mRNA expression in BAL fluid and lungs of RictorKO asthma mice was significantly lower, as detected by quantitative PCR (P<.01, Figure 4H). Moreover, the freshly isolated $CD4^+$ T cells from either the BAL fluid or the spleens of RictorKO allergic airway inflammation mice expressed significantly less IL-9, as detected by flow cytometry (P<.01, Figure 4I,J; P<.001, Figure 4K,L). Thus, all our observations support that T-cell-specific Rictor deficiency impairs Th9 polarization in vivo and reduces Th9-mediated allergic pathogenesis.

3.5 | Rictor-deficient Th9 cells mediate less severe allergic airway inflammation upon adoptive transfer

Given the fact that RictorKO mice exhibit resistance to allergic airway inflammation induced by OVA, we asked whether these effects are mediated by the poor Th9 cell differentiation and function. To

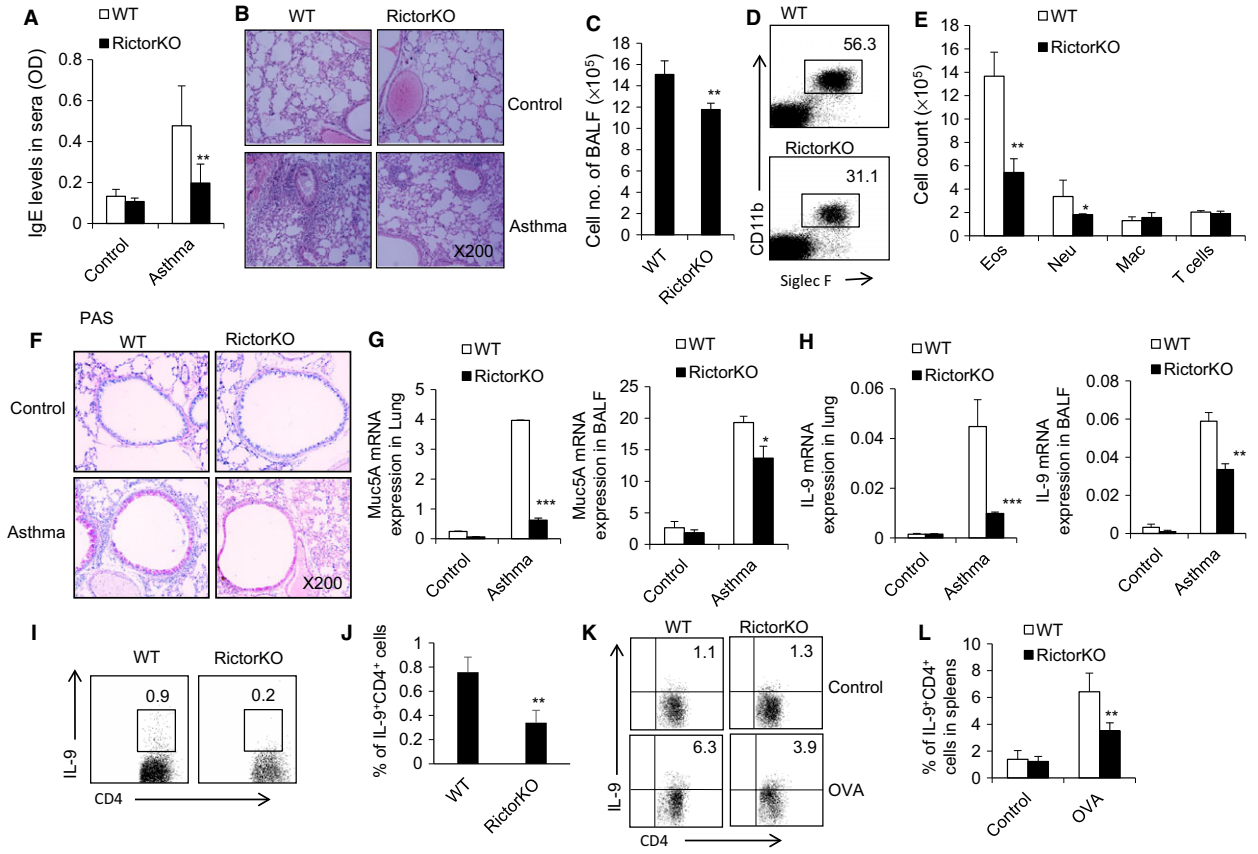

FIGURE 4 Mice with Rictor deficiency specifically in T cells are resistant to the development of allergic inflammation. (A) ELISA analysis of OVA-specific IgE level in the sera of WT or RictorKO mice before or after ovalbumin (OVA) challenge. (B) Representative H&E staining of lung tissues of OVA-challenged or control WT and RictorKO mice. Ovalbumin-presensitized WT and RictorKO mice were challenged with aerosolized OVA daily for 5 days, and the assays were performed on day 6 after OVA challenge. (C) BALF cell numbers in RictorKO and WT mice after OVA challenge. (D) The infiltrated $CD11b^{+}Siglec\ F^{+}$ granulocytes in BALF of WT and Rictor-deficient mice were assayed by flow cytometry. (E) Absolute cell numbers in BALF of WT and RictorKO mice after OVA challenge. Eos, eosinophils; Neu, neutrophils; Mac, macrophages. (F) Staining for mucus production with periodic acid-Schiff reagent (PAS) of WT and RictorKO mice. Original magnification, ×200. (G) The mRNA expressions of Muc5A in bronchoalveolar lavage (BAL) fluid and Lung tissue of WT and Rictor-deficient OT II mice were detected by real-time PCR. (H) The mRNA expressions of IL-9 in BAL fluid and Lung tissue of WT and Rictor-deficient mice were detected by real-time PCR. (I) Flow cytometry analysis of IL-9^{+} $CD4^{+}$ T cells in draining lymph node cells of WT and RictorKO mice after ovalbumin (OVA) challenge. (J) The percentages of IL-9^{+} $CD4^{+}$ T cells in draining lymph node cells of WT and RictorKO mice after OVA challenge. (K) Flow cytometry analysis of IL-9^{+} $CD4^{+}$ T cells in spleens of WT and RictorKO mice after OVA challenge. (L) The percentages of IL-9^{+} $CD4^{+}$ T cells in spleens of WT and RictorKO mice after OVA challenge. Data are shown as mean±SD (n=5), which represent two or three independent experiments. *P<.05, **P<.01, and ***P<.001 compared with WT cells or the controls. [Colour figure can be viewed at wileyonlinelibrary.com]

address this issue, we used an allergic airway inflammation mouse model by adoptive transfer of Th9 cells induced in vitro, in which naïve syngeneic WT mice received Th9-polarized WT or RictorKO TCR-transgenic OTII $CD4^{+}$ Th9 cells and then challenged with aerosolized OVA as described in materials and methods. Administration of Rictor-deficient OTII Th9 cells induced significantly reduced severity of allergic airway inflammation as evidenced by pathological assessment (Figure 5A). This was further confirmed by much less infiltration of leukocytes and eosinophils in the BAL fluid in mice received Rictor-deficient OTII Th9 cells than mice received WT OTII Th9 cells (P<.001, Figure 5B-D). In addition, PAS staining demonstrated that mice received Rictor-deficient OTII Th9 cells produced less mucus in bronchus of lung tissues (Figure 5E). The mRNA expressions of *Muc5ac* and *Il-9* were significantly decreased in the lung tissues and the cells of BAL fluid of asthma-induced mice with Rictor-deficient OTII Th9 cell transfer than mice received WT OTII Th9 cells (P<.01, Figure 5F,G). Meanwhile, the isolated $CD4^{+}$ T cells from draining lymph nodes (dLNs) of allergic airway inflammation mice received Rictor-deficient OTII Th9 cells expressed less IL-9 molecules than those in mice received WT OTII Th9 cells as detected by flow cytometry assays (P<.01, Figure 5H). In parallel, significantly less TCRVα2+ CD4+ T cells (OTII cells) were detected in allergic airway inflammation mice received Rictor-deficient OTII Th9 cells than those in mice received WT OTII Th9 cells as detected by flow cytometry assays (P<.01, Figure 5I,J). Taken together, the decreased Th9 cell differentiation and function by Rictor deficiency significantly ameliorate the pathogenesis of allergic airway inflammation in mice.

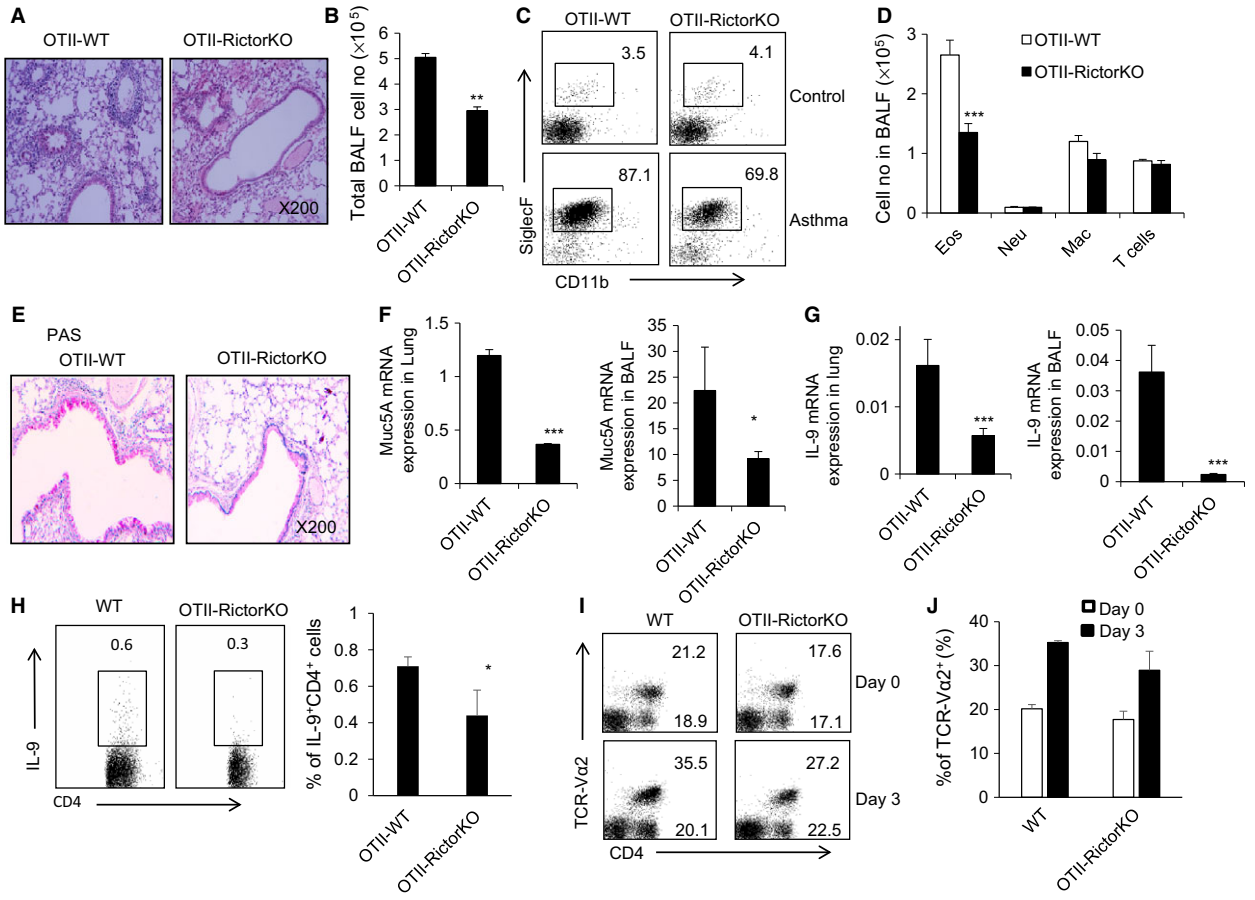

FIGURE 5 Rictor-deficient Th9 cells mediate less severe allergic inflammation upon adoptive transfer. WT and Rictor-deficient OTII $CD4^+$ T cells were polarized in vitro under Th9 conditions and transferred into syngeneic WT recipients before daily i.n. challenges with ovalbumin (OVA) for 5 days and subsequent analysis of pulmonary inflammation. (A) Airway histology (H&E) of lung tissues from mice treated as indicated. (B) Total cell numbers from the BALF (average±SEM of 4-5 mice). (C) The infiltrated $CD11b^+$ Siglec F^+ granulocytes in BALF of recipient mice received WT and Rictor-deficient OT II Th9 cells were assayed by flow cytometry. (D) Cell numbers in BALF of recipient mice received WT and Rictor-deficient OT II Th9 cells were assayed. Eos, eosinophils; Neu, neutrophils; Mac, macrophages. (E) The staining for mucus production with periodic acid-Schiff reagent (PAS) of the lung tissues of recipient mice received WT and Rictor-deficient OT II Th9 cells were assayed. Original magnification, ×200. (F) The mRNA expressions of Muc5A in the lungs and BALF of recipient mice received WT and Rictor-deficient OT II Th9 cells were detected by real-time PCR. (G) The mRNA expressions of IL-9 in the lungs and BALF of recipient mice received WT and Rictor-deficient OT II Th9 cells were detected by real-time PCR. (H) Flow cytometry analysis of IL-9+CD4+ cells in draining lymph node cells of recipient mice received WT and Rictor-deficient OT II Th9 cells. (I) Flow cytometry analysis of TCRVα2$^+$CD4$^+$ T cells (OTII cells) in draining lymph node cells of recipient mice received WT and Rictor-deficient OT II Th9 cells after OVE challenge. Data are shown as mean±SD (n=5), representing one of three independent experiments. *P<.05, **P<.01, and ***P<.001 compared with the controls. [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

In the present study, we have uncovered a novel role of mTORC2 in regulating IL-9 production and mTORC2 deficiency in T cells leads to less severe inflammation in the mouse allergic airway inflammation model.

The mTORC1 complex has been well shown to impact Th1, Th2, Th17, and iTreg differentiation. Deletion of Rheb in T cells leads to largely impaired Th1 and Th17 polarization, but Th2 differentiation remains unchanged.[37] Interestingly, ablation of Raptor, the core component of mTORC1, also impairs Th2 differentiation.[33] These observations suggest that distinct components of mTORC1 signaling pathway might have differential effects on T cell biology, even though they share multiple biological functions in common. On the other hand, it is known that mTORC2 and SGK1 are both required for Th2 differentiation.[37,38] However, it remains unexplored regarding the role of mTORC2 in Th9 cell differentiation and function.

Th9 cells represent the recently described effector lineage that secret a large amounts of IL-9. Our understanding toward Th9 biology is still evolving, and no data suggest the link between mTOR signaling and Th9 differentiation thus far. T-bet, GATA3, RORγt, and Foxp3 represent the master lineage defining transcription factors driving Th1, Th2, Th17, and Treg differentiation, respectively. On the other hand, STAT6, GATA3, Smad3, IRF4, and PU.1 have been shown to regulate IL-9 expression. However, none of these transcription factors alone defines the Th9 lineage differentiation.

Instead, it seems TCR, IL-4, and TGF-β work collaboratively to establish the transcriptional network which thus drives Th9 gene core signature expression. Here, we show that mTORC2 deficiency impairs Th9 differentiation in addition to the defective Th2 development. The sorted RictorKO naïve $CD4^+$ T cells proliferated efficiently and expressed comparable levels of activation markers like CD25 and CD69 after anti-CD3/CD28 mAb stimulation as WT naïve $CD4^+$ T cells. Rictor naïve $CD4^+$ T cells differentiate into Th1 cells efficiently as WT naïve $CD4^+$ T cells as well. Thus, the poor Th9 differentiation of RictorKO naïve $CD4^+$ T cells is unlikely due to T-cell activation failure. The deficient differentiation of Th9 cells from the sorted RictorKO naïve $CD4^+$ T cells indicated that mTORC2 intrinsically regulates Th9 cell differentiation.

Given the decreased Akt activity due to mTORC2 deficiency, indeed, we demonstrate that Akt inhibition also reduce Th9 differentiation in vitro. Moreover, we observed decreased Foxo1/Foxo3a phosphorylation in the CD4 T cells lacking mTORC2. This decreased Foxo1/Foxo3a phosphorylation leads to their nuclear retention, which show enhanced transcriptional activity. However, either Foxo3a deletion or knocking down Foxo1 in T cells induces defective Th9 differentiation, which excludes the possibility of Foxo1/Foxo3a in mediating the effects of mTORC2 on IL-9 production. SGK1 and PKC-θ represent two other key targets downstream of mTORC2. It has been shown that SGK1 controls Th2 differentiation through regulating the expression of Tcf-1.[38] Interestingly, overexpression of constitutive active PKC-θ mutants rescued the defective Th2 differentiation in the absence of Rictor.[36] In this regard, it remains further exploration regarding the role of SGK1 and PKC-θ in Th9 differentiation in the context of mTORC2 deficiency.

Asthma is a chronic inflammatory disease of the airways characterized by infiltration of eosinophils, basophils, mast cells, and $CD4^+$ Th cells into the airway submucosa, which are important cells underlying allergic eosinophilic asthma.[2,3] In addition, the role of IL-9 in allergic asthma is currently recognized. Adoptively transferred Th9 cells lead to allergic airway disease characterized by increased amounts of eosinophil recruitment, tissue mast cell numbers, and serum IgE following OVA challenge.[13-15,41] IL-9 expression is higher in lungs of asthmatic patients, and transgenic expression of IL-9 results in allergic inflammation.[5,6,8,9] IL-9 promotes mucus production from lung epithelial cells and pulmonary mastocytosis.[10,11] Intriguingly, we have uncovered that mTORC2 deficiency impairs Th9 differentiation by reducing IRF4 expression or activity, which was recently demonstrated in alternatively activated macrophages.[45] In our case, the decreased IRF4 expression was likely due to the dual effects of decreased STAT6 and Akt activity in the absence of mTORC2. It remains to be determined how STAT6 activity was reduced in response to IL-4 stimulation in Rictor-deficient T cells under Th9 differentiation and whether certain specific member of suppressor of cytokine signaling components were involved in this regulation. Altogether, our observations support the fact that targeting mTORC2/Akt/IRF4 axis may have important implications for the treatment of allergic asthma.

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AUTHOR CONTRIBUTIONS

H.C. and L.Z. (Lianjun Zhang) designed, carried out the experiments, analyzed the data, and wrote the manuscript; P.W. and H.S. carried out the experiments, analyzed the data, and revised the manuscript; W.W. analyzed data and provided comments; Z.C. typed the genetically modified mice, performed flow cytometry assays; L.Z. (Lianfeng Zhang) provided animal models and revised the manuscript; X.Z. analyzed data, revised the manuscript, and provided comments. Y.Z. designed experiments, analyzed data, wrote the manuscript, and provided overall supervision.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Chung KF. Targeting the interleukin pathway in the treatment of asthma. *Lancet*. 2015;386:1086-1096.
2. Barnes PJ. Therapeutic approaches to asthma-chronic obstructive pulmonary disease overlap syndromes. *J Allergy Clin Immunol*. 2015;136:531-545.
3. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med*. 2012;18:673-683.
4. Schuijs MJ, Willart MA, Hammad H, Lambrecht BN. Cytokine targets in airway inflammation. *Curr Opin Pharmacol*. 2013;13:351-361.
5. Shimbara A, Christodoulopoulos P, Soussi-Gounni A, et al. IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. *J Allergy Clin Immunol*. 2000;105:108-115.
6. Erpenbeck VJ, Hohlfeld JM, Volkmann B, et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. *J Allergy Clin Immunol*. 2003;111:1319-1327.
7. Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. *J Exp Med*. 1998;188:1307-1320.
8. Temann UA, Ray P, Flavell RA. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. *J Clin Invest*. 2002;109:29-39.
9. Forbes EE, Groschwitz K, Abonia JP, et al. IL-9- and mast cell-mediated intestinal permeability predisposes to oral antigen hypersensitivity. *J Exp Med*. 2008;205:897-913.

10. Townsend JM, Fallon GP, Matthews JD, Smith P, Jolin EH, McKenzie NA. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. *Immunity*. 2000;13:573-583.
11. Cheng G, Arima M, Honda K, et al. Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. *Am J Respir Crit Care Med*. 2002;166:409-416.
12. Chang HC, Sehra S, Goswami R, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol*. 2010;11:527-534.
13. Staudt V, Bothur E, Klein M, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity*. 2010;33:192-202.
14. Brustle A, Heink S, Huber M, et al. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol*. 2007;8:958-966.
15. Jones CP, Gregory LG, Causton B, Campbell GA, Lloyd CM. Activin A and TGF-beta promote T(H)9 cell-mediated pulmonary allergic pathology. *J Allergy Clin Immunol*. 2012;129:1000-1010.
16. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4 + T cells. *Science*. 2010;327:1098-1102.
17. Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol*. 2014;35:61-68.
18. Perumal NB, Kaplan MH. Regulating Il9 transcription in T helper cells. *Trends Immunol*. 2011;32:146-150.
19. Yang XO, Zhang H, Kim BS, et al. The signaling suppressor CIS controls proallergic T cell development and allergic airway inflammation. *Nat Immunol*. 2013;14:732-740.
20. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. *Cancer Cell*. 2007;12:9-22.
21. Betz C, Hall MN. Where is mTOR and what is it doing there? *J Cell Biol*. 2013;203:563-574.
22. Bentzinger CF, Romanino K, Cloetta D, et al. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. *Cell Metab*. 2008;8:411-424.
23. Pollizzi KN, Patel CH, Sun IH, et al. mTORC1 and mTORC2 selectively regulate CD8 + T cell differentiation. *J Clin Invest*. 2015;125:2090-2108.
24. Zhang L, Tschumi BO, Lopez-Mejia IC, et al. Mammalian target of rapamycin complex 2 controls CD8 T cell memory differentiation in a Foxo1-dependent manner. *Cell Rep*. 2016;14:1206-1217.
25. Chi H. Regulation and function of mTOR signalling in T cell fate decisions. *Nat Rev Immunol*. 2012;12:325-338.
26. Zhu L, Yang T, Li L, et al. TSC1 controls macrophage polarization to prevent inflammatory disease. *Nat Commun*. 2014;5:4696.
27. Chen H, Zhang L, Zhang H, et al. Disruption of TSC1/2 signaling complex reveals a checkpoint governing thymic CD4 + CD25 + Foxp3 + regulatory T-cell development in mice. *FASEB J*. 2013;27:3979-3990.
28. Zhang L, Zhang H, Li L, et al. TSC1/2 signaling complex is essential for peripheral naive CD8 + T cell survival and homeostasis in mice. *PLoS One*. 2012;7:e30592.
29. Powell JD, Pollizzi KN, Heikamp EB, Horton MR. Regulation of immune responses by mTOR. *Annu Rev Immunol*. 2012;30:39-68.
30. Waickman AT, Powell JD. mTOR, metabolism, and the regulation of T-cell differentiation and function. *Immunol Rev*. 2012;249:43-58.
31. Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity*. 2009;30:832-844.
32. Scholz G, Jandus C, Zhang L, et al. Modulation of mTOR signalling triggers the formation of stem cell-like memory T cells. *EBioMedicine*. 2016;4:50-61.
33. Yang K, Shrestha S, Zeng H, et al. T cell exit from quiescence and differentiation into Th2 cells depend on Raptor-mTORC1-mediated metabolic reprogramming. *Immunity*. 2013;39:1043-1056.
34. Zhang L, Tschumi BO, Corgnac S, et al. Mammalian target of rapamycin complex 1 orchestrates invariant NKT cell differentiation and effector function. *J Immunol*. 2014;193:1759-1765.
35. Guertin DA, Stevens DM, Thoreen CC, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. *Dev Cell*. 2006;11:859-871.
36. Lee K, Gudapati P, Dragovic S, et al. Mammalian target of rapamycin protein complex 2 regulates differentiation of Th1 and Th2 cell subsets via distinct signaling pathways. *Immunity*. 2010;32:743-753.
37. Delgoffe GM, Pollizzi KN, Waickman AT, et al. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat Immunol*. 2011;12:295-303.
38. Heikamp EB, Patel CH, Collins S, et al. The AGC kinase SGK1 regulates TH1 and TH2 differentiation downstream of the mTORC2 complex. *Nat Immunol*. 2014;15:457-464.
39. Sun B, Hu X, Liu G, et al. Phosphatase Wip1 negatively regulates neutrophil migration and inflammation. *J Immunol*. 2014;192:1184-1195.
40. Wu TT, Sun CM, Chen ZG, et al. Smad3-deficient CD11b(+)Gr1(+) myeloid-derived suppressor cells prevent allograft rejection via the nitric oxide pathway. *J Immunol*. 2012;189:4989-5000.
41. Niedbala W, Besnard AG, Nascimento DC, et al. Nitric oxide enhances Th9 cell differentiation and airway inflammation. *Nat Commun*. 2014;5:4575.
42. Abreu SC, Antunes MA, Mendonca L, et al. Effects of bone marrow mononuclear cells from healthy or ovalbumin-induced lung inflammation donors on recipient allergic asthma mice. *Stem Cell Res Ther*. 2014;5:108.
43. Huang XF, Tang L, Wang F, Song GQ. Astragaloside IV attenuates allergic inflammation by regulation Th1/Th2 cytokine and enhancement CD4(+)CD25(+)Foxp3 T cells in ovalbumin-induced asthma. *Immunobiology*. 2014;219:565-571.
44. Groneberg DA, Eynott PR, Oates T, et al. Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung. *Respir Med*. 2002;96:81-86.
45. Huang SC, Smith AM, Everts B, et al. Metabolic reprogramming mediated by the mTORC2-IRF4 signaling axis is essential for macrophage alternative activation. *Immunity*. 2016;45:817-830.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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