**The Kinase Complex mTOR Complex 2 Promotes the Follicular Migration and Functional Maturation of Differentiated Follicular Helper CD4+ T Cells During Viral Infection**

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Follicular helper CD4+ T (Tfh) cells are critical for optimal B-cell-mediated humoral immunity by initiating, fueling, and sustaining germinal center reactions. The differentiation of Tfh cells relies on multiple intrinsic and extrinsic factors; however, the details by which these factors are integrated to coordinate Tfh differentiation are largely unknown. In this study, using a mouse model of acute lymphocytic choriomeningitis virus (LCMV) viral infection, we demonstrate that mTOR complex 2 (mTORC2) kinase integrates TCR signaling and ICOS-mediated co-stimulation to promote late differentiation and functional maturation of virus-specific Tfh cells. Specifically, mTORC2 functions to maintain Tfh lineage specifications, including phenotypes, migratory characteristics, and functional properties. Thus, our results highlight the importance of mTORC2 in guarding Tfh phenotypic and functional maturation.

Keywords: mechanistic target of rapamycin complex 2, follicular helper T cells, germinal center, B cells, acute viral infection

**INTRODUCTION**

Effective humoral immunity protects individuals from invading pathogens by producing high-affinity, class-switched antibodies, which require cooperation between pathogen-specific B cells and follicular helper CD4+ T (Tfh) cells. Tfh cells were originally defined as a unique helper CD4+ subset characterized by high expression of the chemokine receptor CXCR5, which facilitates Tfh cell migration toward B cell follicles (1–6), where they interact with and assist cognate B cells. Tfh cells provide essential signals to B cells, including engagement of the inducible T cell co-stimulator ICOS, the ligand for the costimulatory receptor CD40, T cell inhibitory receptor PD-1, interleukin 21 (IL-21), and interleukin 4 (IL-4) (7), leading to the formation and maintenance of germinal centers (GCs), in which B cells undergo somatic hypermutation, antibody affinity maturation, and final differentiation into long-lived memory B cells and plasma cells (8).

Follicular helper CD4+ T cell differentiation is a multistage process that is tightly controlled by multiple factors (7). In acute viral infection, dendritic cells (DCs) initiate Tfh programming by priming antigen-specific CD4+ T cells for activation (9). The activated CD4+ T cells, with upregulated...
CXCR5 and Bcl-6 but downregulated Blimp-1 expression, differentiate toward T_{FH} cells (10–12). The T_{FH} precursors are generated as early as 2 days after activation (13), which has recently been proven to be mediated by the transcription factor T cell factor-1 (TCF-1) and achaete scute homolog-2 (ASCL2) (14–17). TCF-1 promotes early T_{FH} cell differentiation by upregulating Bcl-6 but repressing Blimp1 expression (1,4), while ASCL2 participates in early T_{FH} induction by increasing the follicular homing ability of T_{FH} cells (17). These early differentiated T_{FH} cells subsequently migrate to the T–B cell border and acquire full polarization into GC T_{FH} cells with maximal functions upon engagement of B cells (18–21). Of note, initiation of T_{FH} differentiation requires priming from DCs but not B cells (7, 9, 22, 23), while full commitment and maintenance of T_{FH} cells depend on the presence of cognate B cells (7, 18, 23). Unlike early T_{FH} early polarization, underlying mechanisms during complete differentiation and functional maturation of T_{FH} cells in the B cell-dependent phase are poorly understood.

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that is involved in diverse cellular processes, including cell growth, proliferation, differentiation, metabolism, and survival, by sensing and integrating environmental cues (24). The mTOR kinase exists in two distinct complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are defined by scaffolding subunit regulatory-associated protein of mTOR (Raptor) and raptor-miycin-insensitive companion of mTOR (Rictor), respectively (25, 26). mTORC1 has been reported to regulate protein translation and glucose and lipid metabolism by phosphorylating the downstream targets ribosomal protein S6 kinase and elf4E-binding protein 1 (4E-BP1) (27–29). mTORC2 mainly engages in the regulation of cell survival, metabolism, and cytoskeletal organization by phosphorylating many AGC kinases, including AKT (at position Ser473), SGK1, and PKC-α (30). Both mTORC1 and mTORC2 have been reported to participate in a variety of T cell immune responses (31). mTORC1 signaling promotes the differentiation of T_{FH}1 and T_{FH}17, whereas it inhibits the differentiation and suppressor functions of T_{FH} cells (32–37). Additionally, the interleukin-2 (IL-2)-mTORC1 signaling axis promotes T_{FH}1 but inhibits T_{FH} cell differentiation to orchestrate the reciprocal balance between T_{FH}1 and T_{FH} cell fates during acute viral infection (38). Additionally, mTORC1 has been reported to regulate follicular regulatory T (T_{FR}) cell differentiation from conventional regulatory T cells (39). In contrast to mTORC1, the knowledge about mTORC2 in the regulation of T cell immunity is quite limited. It is known that mTORC2 signaling favors T_{FH}2 differentiation (32, 40). Recently, two groups have reported that mTORC2 is essential for T_{FH} cell differentiation at the steady state in Peyer’s patches (PPs) and upon protein immunization or viral infection (41, 42). However, it remains unknown whether mTORC2 selectively regulates early fate commitment or later lineage maintenance of T_{FH} cells, or both. Additionally, whether mTORC2 regulates the effector functions of differentiated T_{FH} cells remains to be investigated.

Here, we investigate the role of mTORC2 in regulating T_{FH} differentiation at early and late stages, as well as the effector function of T_{FH} cells in response to acute viral infection. We use lymphocytic choriomeningitis virus (LCMV) to establish a mouse model of acute viral infection, in which virus - specific CD4+ T cells primarily differentiate into T_{FH} and T_{FH}1 effector cells (43). Our findings demonstrate that mTORC2 signaling is selectively critical for T_{FH} differentiation in the late stage (4–8 days), but not early fate commitment (1–3 days). Moreover, mTORC2 plays an essential role in mediating the effector function of T_{FH} cells to assist B cells, which is accomplished by regulating the T_{FH} transcriptional program and migratory ability toward B cell follicles.

**MATERIALS AND METHODS**

**Mice, Virus, and Immunization**

*Rictor*Δ5, Cdh4-Cre transgenic, and C57BL/6J (CD45.2*+* and CD45.1*+*) mice were obtained from the Jackson Laboratory. *Sh2d1a*−/− (Sap−/−) mice were provided by Dr. Hai Qi (Tsinghua University). SMARTA (CD45.1*+) transgenic mice and the LCMV Armstrong strain were provided by Dr. Rafi Ahmed (Emory University). The mice were infected with 2 × 10^6 plaque-forming units (PFU) of LCMV Armstrong at 6–10 weeks of age, and both sexes were included without randomization or “blinding.” Bone marrow (BM) chimeras were infected after 8–10 weeks of reconstitution. To establish bacterial infection, mice were intravenously infected with 1 × 10^6 colony-forming units of *Listeria monocytogenes* expressing LCMV glycoprotein-specific I-Ab-restricted CD4+ T cell epitope gp61-80 (L-M-gp61), that was created from vector strain1 (+4). 4-Hydroxy-3-nitrophenylacetyl-conjugated ovalbumin (NP-OVA) (N-5051-100, Biosearch Technology) was 1:1 emulsified with Complete Freund’s Adjuvants (F5881, Sigma) and immunized mice subcutaneously of 100 μg per mouse. All immunized mice were housed in accordance with institutional biosafety regulations of the Third Military Medical University. All mouse experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of the Third Military Medical University.

**Flow Cytometry and Antibodies**

Major histocompatibility complex class II (I-A*) tetramer specific for the LCMV epitope of glycoprotein amino acids 66–77 was provided by the tetramer core facility of the US National Institutes of Health (Emory). The antibodies used for flow cytometry are listed in Table S1 in Supplementary Material. Surface staining was performed in PBS containing 2% FBS. CXCR5 staining was performed using purified anti-CXCR5 (BD Biosciences) for 1 h at 4°C, followed by biotinylated anti-rat immunoglobulin G (IgG) (Jackson Immunoresearch) and then fluorescently labeled streptavidin (eBioscience) for 30 min on ice. Staining was performed in PBS containing 0.5% BSA, 2% FCS, and 2% normal mouse serum. Staining for Bcl-6, c-Maf, TCF-1, IgG1, IgG2a, and Foxp3 was performed with the Foxp3/Transcription Factor Staining Buffer Set (00-5523, eBioscience). Major histocompatibility complex class II tetramer staining was performed by incubation of the tetramer with cells for 1 h at 37°C. For detection of phosphorylated mTOR signaling proteins, lymphocytes were first stained with surface markers and then were stimulated with anti-CD3 (2 μg/ml, 100302, Biolegend),
anti-CD28 (0.5 μg/ml, 102102, Biolegend), anti-ICOS (2 μg/ml, 14-9949-82, eBioscience), gp61–80 peptide (2 μg/ml), or CXCL13 (4 μg/ml, 4583906, Biolegend) at 37°C for 1 h. Stimulated cells were immediately fixed with Phosflow Lyse/Fix buffer (558049, BD Biosciences), followed by permeabilization with Phosflow Perm buffer 1 (557885, Biosciences) and staining with primary unconjugated antibodies against p-S6 (Ser 235/236) (D57.2.2E, Cell Signaling Technology) and p-AKT (Ser 473) (#40608, Cell Signaling Technology). Next, primary unconjugated antibodies were detected by secondary staining with anti-rabbit IgG A488 antibody (A21206, Invitrogen) or anti-rabbit IgG A647 antibody (#4414S, Cell Signaling Technology). Flow cytometry data were acquired with a FACS Canto II (BD Biosciences) and were analyzed with FlowJo software (Tree star, Ashland, OR, USA).

Retroviral Constructs and Transduction
The humanized-Cre (hCre) coding sequences were amplified and cloned into the vectors MIGR1 (MSCV-IRES-GFP). Retroviruses were packaged by transfection of plasm-E cells with the retroviral vectors along with plasmid pCL-ccl. SMARTA cells were activated in vivo by injection of 200 μg of peptide (LCMV glycoprotein amino acids 61–80) into SMARTA mice. After 18 h, activated SMARTA cells were purified by negative selection with BeaverBeads Mag500 Streptavidin Matrix (22302, Beaver) and then “spin-infected” for 90 min at 37°C by centrifugation with BeaverBeads Mag500 Streptavidin Matrix (22302, Beaver) and then “spin-infected” for 90 min at 37°C by centrifugation (800 × g) with freshly harvested retrovirus supernatants, 8 μg/ml polybrene (H9268, Sigma-Aldrich), and 20 ng/ml of IL-2 (130-098-221, Miltenyi Biotec). Then, the transduced SMARTA cells were transferred into recipient mice, followed by infection of the hosts with LCMV.

Adoptive Transfer
A total of 1 × 10⁶ (for analysis at day 3) or 2 × 10⁴ (for analysis at day 8) retrovirus-transduced SMARTA (CD45.1⁺) cells were adoptively transferred into naive C57BL/6J (CD45.2⁻) mice, which were infected intravenously with 2 × 10⁶ PFU (day 3) or infected intraperitoneally with 2 × 10⁶ PFU (day 8) of the LCMV Armstrong strain on the following day. For assessment of mTOR activity kinetics, a total of 2 × 10⁵ (for analysis at day 2) or 4 × 10⁴ (for analysis at day 5) or 2 × 10⁴ (for analysis at day 8) naive SMARTA (CD45.1⁺) cells were adoptively transferred into naive C57BL/6J (CD45.2⁺) mice, which were infected intravenously with 2 × 10⁶ PFU (for analysis at day 2) or infected intraperitoneally with 2 × 10⁵ PFU (for analysis at day 5) and 8 of the LCMV Armstrong strain on the following day. For evaluation of Tfh cell function, 3 × 10⁶ sorted Tfh cells from Rictor⁻/⁻ or WT mouse (CD45.1⁺) were adoptively transferred into Sap⁻/⁻ recipient mice (CD45.2⁺) which were infected with LCMV 1 day before cell transfer and then the hosts were analyzed on day 6 after cell transfer.

Enzyme-Linked Immunosorbent and Enzyme-Linked Immunospot Assay
Lymphocytic choriomeningitis virus-specific IgG and antibody-secreting cells (ASCs) were measured by enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISPOT) assay, respectively, which has been described (45, 46).

Generation of Bone Marrow Chimeras
For each chimera, 5 × 10⁶ BM cells of a 4:6 mixture derived from Rictor⁻/⁻ or Rictorfl/fl (CD45.2⁺) mice and C57BL/6J (CD45.1⁺) mice were intravenously transferred into lethally irradiated (2 doses of 550 rads each) C57BL/6J (CD45.1⁺) recipients. Recipient mice were allowed 8–10 weeks for reconstitution before infection with LCMV.

Immunofluorescence Staining
Tissues immersed in OCT were quickly frozen in liquid nitrogen and cut into 7-µm-thick sections. Frozen tissue sections were fixed in cold acetone for 10 min at −20°C, blocked with 5% BSA and 1:100 Fc-blocker in PBS, and stained with biotin-IgD, FITC-labeled anti-GL7, and PE-labeled anti-CD4, followed by Alexa 650 dye-labeled avidin. After each step, the slides were washed at least three times with PBS. Coverslips were mounted on slides using an antifade kit (BOSTER) and then examined using a Zeiss LSM 800 confocal fluorescence microscope. The images were processed with LSM Image Examiner software (Zeiss).

Microarray and Bioinformatics Analysis
Isolation of Tfh cells from WT and Rictor⁻/⁻ mice at day 8 after infection has been described previously (14). Total RNA was extracted according to the TRIzol reagent protocol (Life Technologies) and submitted to CapitalBio for microarray analysis. Gene-set-enrichment analysis (GSEA) software (Broad Institute) was used for analysis (47). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GSE111536) and are accessible through GEO Series accession number GSE111536 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111536).

Quantitative RT-PCR
For comparison of gene expression in Tfh cells from Rictor⁻/⁻ and WT mice, the cells were sorted and subsequently lysed in TRIzol LS reagent (10926; Life Technologies). Total RNA was extracted and reverse-transcribed with a RevertAid H Minus First-Strand cDNA Synthesis Kit (K1632; Thermo Scientific). The resulting cDNA was analyzed for expression of various genes with the SYBR Green PCR kit (208054, QIAGEN) on a CFX96 Touch Real-Time System (Bio-Rad) and the appropriate primers for “test genes” (Table S2 in Supplementary Material).

Transwell Migration Chemotaxis Assay
For enrichment of CD4⁺ T cells, total splenocyte samples from WT and Rictor⁻/⁻ mice at day 8 after infection with LCMV were subjected to depletion of cells that were positive for lineage markers (Lin⁺ cells) using biotin-conjugated antibodies [anti-CD8 (53–6.7), anti-B220 (RA3-6B2), anti-CD11c (N418), anti-Gr-1 (RB6-8C5), anti-TER119 (TER-119), and anti-NK1.1 (PK136), all from Biolegend] coupled to the BeaverBeads Mag500 Streptavidin Matrix (22302, Beaver). The surfaces of the Lin⁺ cells were then stained with anti-CD4, anti-CD44, anti-GITR, anti-CD25, and anti-CCR5 to identify Tfh cells. Next, 4 × 10⁶ Tfh cells from WT or Rictor⁻/⁻ mice were loaded into the upper chamber of a 24-well transwell plate (5-µm pore, Corning), and 600 μl of chemotaxis
medium supplemented with or without the CXCL13 (4 µg/ml, 4583906, Biolegend) was added to the lower chamber. The cells were allowed to migrate for 3 h in a 5% CO2 incubator at 37°C. Then, all the migrated cells were collected from the lower chamber, and the numbers of migrated T\(_{FH}\) cells were determined by flow cytometry (FACS Canto II). Based on the absolute number of T\(_{FH}\) cells, the “net migration (% of input)” was calculated as follows: Net migration (% of input) = (# of migrated T\(_{FH}\) cells to CXCL13 – # of migrated T\(_{FH}\) cells in the absence of CXCL13) (# of T\(_{FH}\) cells in the input sample).

Conjugate Adhesion Assay

B cells were activated in vivo by injection of 30 µg of LPS (ALX-581-008-Loo2, Enzo Life Sciences) into naive C57BL/6J mice and purified by negative selection with magnetic beads (22302, Beaver) after 18 h. Rictor\(^{fl/fl}\)-Cd4-Cre-SMARTA (Rictor\(^{-/-}\) SMARTA) cells and WT SMARTA (CD45.1\(^{+}\)) cells were adoptively transferred into naive C57BL/6J (CD45.2\(^{+}\)) mice, following intraperitoneal injection with 2 × 10\(^5\) PFU of the LCMV Armstrong strain. Rictor\(^{-/-}\) and WT SMARTA T\(_{FH}\) cells were sorted from Rictor\(^{-/-}\) SMARTA and WT SMARTA chimeras, respectively, at day 8 after LCMV infection. Then, 2 × 10\(^5\) Rictor\(^{-/-}\) and WT SMARTA T\(_{FH}\) cells were incubated for 1 h at 37°C in 96-well U-bottom plate with 8 × 10\(^3\) LPS-activated B cells pulsed with gp61–80 peptide (LCMV glycoprotein amino acids 61–80). The frequency of T cell–B cell conjugates was quantified by flow cytometry as CD4\(^+\)B220\(^-\). I-A\(^e\) gp66-77 tetramer\(^\dagger\) T\(_{FH}\) cells were also used to perform the adhesion assay, and 5 × 10\(^4\) I-A\(^e\) gp66-77 tetramer\(^\dagger\) T\(_{FH}\) cells were incubated for 1 h at 37°C in 96-well U-bottom plate with 2 × 10\(^5\) LPS-activated B cells pulsed with gp61-80 peptide. The frequency of T cell–B cell conjugates was quantified by flow cytometry as I-A\(^e\) gp66-77 tetramer\(^\dagger\) CD4\(^+\) B220\(^-\).

In Vitro T\(_{FH}\) Function Assay

Follicular helper CD4\(^+\) T (T\(_{FH}\)) cells were sorted from WT or Rictor\(^{-/-}\) mice, and B cells were sorted from C57BL/6J mice at day 8 after LCMV infection. U-bottom 96-well plates were seeded with 5 × 10\(^4\) B cells alone, 5 × 10\(^4\) B cells and 3 × 10\(^4\) wild-type T\(_{FH}\) cells or 5 × 10\(^4\) B cells and 3 × 10\(^4\) Rictor\(^{-/-}\) T\(_{FH}\) cells, and then supplemented with 200 µl of RPMI medium (10% FBS, Pen–Strep, l-glutamine) containing anti-CD3 (2 µg/ml, 100302, Biolegend) and anti-IgM (5 µg/ml, 115-006-075, Jackson Immunoresearch). The plates were cultured for 4 days at 37°C, followed by FACS staining using anti-GL7, anti-IgG1, anti-IgG2a, anti-I-A/I-E, anti-CD19, and anti-CD4.

Statistical Analysis

Statistical analysis was conducted with Prism 6.0 software (GraphPad). An unpaired two-tailed t-test with 95% confidence interval was used for calculation of P values. For retroviral transduction and BM chimera experiments, a paired two-tailed t-test with 95% confidence interval was used for calculation of P values. For microarray analysis, we used an unpaired one-tailed t-test with 95% confidence interval for calculation of P values. For in vivo and in vitro T\(_{FH}\) function assay and T cell–B cell adhesion assay, we used the one-way ANOVA with multiple comparisons for calculation of P values.

RESULTS

mTORC2 Signaling Is Elevated in T\(_{FH}\) Cells and Activated by ICOS and CD3

To evaluate the activity of mTORC2 signaling in T\(_{FH}\) and T\(_{H1}\) cells in the context of acute viral infection, we infected wild-type C57BL/6J mice with the Armstrong strain of LCMV and measured the level of AKT phosphorylation at Ser 473, which is an indicator of mTORC2 activity, between T\(_{FH}\) and T\(_{H1}\) cells in the spleen on day 8 post-infection. Flow cytometry data demonstrated that T\(_{FH}\) cells possessed higher mTORC2 activity than T\(_{H1}\) cells upon anti-CD3 plus anti-CD28 stimulation (Figure 1A).

Furthermore, to investigate which stimuli were mainly responsible for mTORC2 activation in T\(_{FH}\) cells, we stimulated splenocytes from wild-type C57BL/6J mice at day 8 of infection with different combinations, including anti-CD3 plus anti-CD28, anti-CD3 plus anti-ICOS, anti-CD3 only, anti-ICOS only, or CXCL13 only. We then compared mTORC2 activity in T\(_{FH}\) cells under these different stimulation conditions and observed that the combination of anti-CD3 plus anti-ICOS elicited the highest mTORC2 signaling (Figure 1B). Next, stimulation of T\(_{FH}\) cells with anti-ICOS alone induced high levels of mTORC2 activity, ranking second only to anti-CD3 plus anti-ICOS (Figure 1B). Moreover, T\(_{FH}\) cells stimulated with either anti-CD3 or anti-CD3 plus anti-CD28 displayed a certain level of mTORC2 activity, while CXCL13 failed to effectively activate mTORC2 (Figure 1B). These results indicated that ICOS and CD3 signaling were pivotal for mTORC2 signaling activation in T\(_{FH}\) cells, while CD28 and CXCL13 signaling might not be necessary. And then, we also compared mTORC2 activity between T\(_{FH}\) and T\(_{H1}\) cells upon anti-CD3 plus anti-ICOS stimulation. Consistently, T\(_{FH}\) cells also exhibited enhanced mTORC2 activity compared with T\(_{H1}\) cells (Figure 1C).

Next, we analyzed the kinetics of mTOR activity in T\(_{FH}\) cells at day 2, 5, and 8 post infection. To achieve this, we adoptively transferred naive SMARTA (CD45.1\(^{+}\)) cells with transgenic TCR specific to LCMV glycoprotein I-A\(^e\) epitope (49) into naive C57BL/6J (CD45.2\(^{+}\)) mice, which were infected with LCMV Armstrong strain on the following day. We found that mTORC2 activity was rapidly induced in early differentiated T\(_{FH}\) cells at 48 h post-infection and subsequently maintained at days 5 and 8 post-infection (Figure 1D). In contrast, mTORC1 was highly induced at the initiation phase of T\(_{FH}\) differentiation but dramatically declined to the baseline later on (Figure 1E). Taken together, these results demonstrated that T\(_{FH}\) cells possessed higher mTORC2 activity compared with T\(_{H1}\) cells. Additionally, ICOS and CD3 signaling might act as important upstream activators of mTORC2 signaling in T\(_{FH}\) cells.

mTORC2 Is Intrinsically Required for Effector T\(_{FH}\) Cell Differentiation

Since mTORC2 activity was upregulated in T\(_{FH}\) cells, we speculated that mTORC2 might play a critical role in T\(_{FH}\) cell responses during acute viral infection. To test this hypothesis, we generated Rictor\(^{fl/fl}\)-Cd4-Cre mice by crossing Rictor\(^{fl/fl}\) mice with transgenic Cd4-Cre mice to conditionally delete Rictor alleles in...
FIGURE 1 | Continued
CD4+ T cells. Next, we infected Rictorfl/flCd4-Cre mice (called “Rictor−/− mice” here) and their Rictorfl/+ littersates (called “WT mice” here) with LCMV Armstrong and measured the level of Rictor mRNA copies and mTORC2 activity in CD4+ T cells. We found that Rictor mRNA expression in both T FH and T H1 cells sorted from Rictor−/− mice was undetectable (Figure S1A in Supplementary Material). Additionally, mTORC2 activity failed to be induced in Rictor−/− CD4+ T cells upon stimulation (Figure S1B in Supplementary Material). However, the level of phosphorylated S6 at Ser 235/236, which is indicative of mTORC1 activity, was comparable between Rictor−/− and WT CD4+ T cells upon stimulation (Figure S1B in Supplementary Material). Therefore, mTORC2 signaling was adequately abrogated in CD4+ T cells from Rictor−/− mice.

Next, we analyzed CD4+ T cell responses at day 8 after LCMV infection, and we found a reduced cell number of total virus-activated CD44hiCD4+ T cells in Rictor−/− mice than control mice (Figure S1C in Supplementary Material) and a similar frequency but lower number of I-Ab restricted LCMV-gp66 epitope-specific CD4+ T cells (Figure 2A). Notably, both the frequency and cell number of tetramer-positive SLAMhiCXCR5+ T FH cells were greatly decreased in Rictor−/− mice, whereas the tetramer-positive SLAMhiCXCR5− T H1 cells showed an increased frequency and similar cell number (Figure 2A). Thus, the reduction of total CD44hiCD4+ T cells and tetramer-positive CD4+ T cells were mainly attributable to a decrease in T FH but not T H1 cells, which was in agreement with a previous study showing that mTORC2 was dispensable for T H1 differentiation (32). Moreover, we also measured the expression of CXCR5, Bcl-6, PD-1, and ICOS in tetramer-positive T FH cells and found that all of these T FH cell-associated molecules were downregulated in Rictor−/− mice compared with the WT control (Figure 2B). In addition to tetramer-positive CD4+ T cells, we also analyzed the responses of bulk activated CD4+ T cell and observed similar phenotypes in Rictor−/− mice (Figure 2C; Figure S1D in Supplementary Material). Notably, we observed an approximately 3-fold lower frequency and 10-fold lower cell numbers of B6εεcMa6B GC T FH cells in Rictor−/− compared with WT mice (Figure 2D), suggesting an impairment of T FH cell maturation in the absence of mTORC2 signaling. These results showed that mTORC2 signaling was essential for T FH differentiation, but dispensable for T H1 differentiation.

However, there was a potential concern that CD8+ T cells in Rictor−/− mice were also mTORC2 signaling deficient, which might impact the viral clearance rate and further confound T FH responses. In addition, the impaired T FH cell differentiation in Rictor−/− mice would result in poorer GC B cell responses, which in turn might negatively influence T FH cell responses as a feedback loop. To more precisely assess the role of mTORC2 signaling in T FH cell responses, we established bone marrow (BM) chimeras by transferring cell mixtures of BM cells derived from Rictor−/− mice (CD45.2+) (40%) and C57BL/6J mice (CD45.1+) (60%) into irradiated C57BL/6J recipients (CD45.1+). Control groups were also generated by transferring BM cell mixtures derived from Rictorfl/+ mice (CD45.2+) (40%) and C57BL/6J mice (CD45.1+) (60%). After 8 weeks of reconstitution, we infected these BM chimeras with LCMV Armstrong and analyzed T FH responses at day 8 after infection. First, we gated on the total CD4+ T cell population, CD4+gp66hi population, and gp66hi T FH and T H1 populations, and then we compared the contribution of CD45.2+ cells derived from Rictor−/− or Rictorfl/+ mice among these populations (Figure 2E). We found that CD45.2+ CD4+ T cells originating from Rictor−/− mice accounted for approximately 22.3% of the total CD4+ T cell population; however, CD45.2+ gp66hi cells of Rictor−/− origin accounted for approximately 15.6% of the total gp66hi T cell subset (Figure 2E). Moreover, Rictor-null CD45.2+ T FH cells contributed approximately 18.4% of the total T FH cells, while CD45.2+ T FH cell derived from Rictor−/− mice contributed only approximately 10.4% of the total T FH cells (Figure 2E). In control BM chimeras, however, CD45.2+ cells of Rictorfl/+ origin exhibited a stable contribution from 52.53 to 55% among the total CD4+ T, CD4+gp66hi, gp66hi T FH, and gp66hi T H1 populations (Figure 2E). Additionally, the expression levels of CXCR5 and Bcl-6 were reduced in Rictor−/− T FH cells, but not Rictorfl/+ T FH cells, compared with the control group (Figure 2F). The results from the BM chimera model collectively illustrated that T FH cell differentiation was specifically dampened in the absence of mTORC2 signaling, and more importantly, mTORC2 was required for T FH differentiation in a cell-autonomous manner.

In addition to LCMV infection, we also analyzed T FH responses in different immunization models by infecting Rictor−/− and WT mice with Listeria monocytogenes expressing a CD4+ T cell epitope derived from LCMV gp61-80 (LM-GP61). Phenotypes were observed on day 8 post-infection. In agreement with the results from the LCMV infection, both gp66hi and activated bulk T FH cells from Rictor−/− mice exhibited decreased frequencies and cell numbers compared with Rictorfl/+ littersates (Figure S2A in Supplementary Material). Additionally, similar results were obtained using the 4-hydroxy-3-NP-OVA immunization model (Figure S2B in Supplementary Material). Thus, mTORC2

**FIGURE 1** | mTOR complex 2 activity is elevated in follicular helper CD4+ T (T FH) cells upon stimulation. (A,C) Comparison of p-AKTThr473 mean fluorescence intensity between T FH (CD44hiCXCR5+ and T FH (CD44hiCXCR5−) cells from the spleen of wild-type C57BL/6J mice at day 8 after lymphocytic choriomeningitis virus infection. (A) T FH and T FH cells were cultured in medium without any stimulus (blank), or stimulated with anti-CD3 and anti-CD28 (CD3+CD28), and the level of p-AKTThr473 was detected by flow cytometry (n = 5 mice per group). (B) T FH cells were activated with different stimuli, and green, red, purple, pink, blue, orange, and gray solid histograms represent the blank, anti-CD3 and anti-CD28, anti-CD3, anti-ICOS, anti-CD3, anti-ICOS, anti-CXCL13, and isotype control, respectively (n = 5 mice per group). (C) T FH and T FH cells were cultured in blank medium or stimulated with anti-CD3 and anti-CD3 and anti-ICOS (CD3+ICOS), and the level of p-AKTThr473 was detected by flow cytometry (n = 5 mice per group). (D,E) Kinetics of mTOR activity in SMARTA T FH cells at day 2, 5, and 8 after infection. (D) SMARTA T FH cells were activated with anti-CD3, anti-ICOS, anti-CD28, and gp61-80 peptide, and then the level of p-AKTThr473 was detected by flow cytometry (n = 5 mice per group). (E) SMARTA T FH cells were activated with anti-CD3, anti-CD28, and gp61-80 peptide, and then the level of p-S6Thr24/20 and p-AKTThr473 was detected by flow cytometry (n = 5 mice per group). ns, not significant, *p < 0.01, **p < 0.001, ***p < 0.0001 [unpaired two-tailed t-test (A,C), one-way ANOVA with multiple comparisons (B,D,E)]. Data are representative of three (A–C) or two (D,E) independent experiments. Error bars are SEM (A–C).
signaling also played a critical role in T<sub>FH</sub> cell responses in both bacterial and protein immunization models.

To exclude the possibility that impaired T<sub>FH</sub> differentiation in Rictor<sup>−/−</sup> mice was due to defects in CD4<sup>+</sup> T cells during T cell development or the naïve state, we assessed the frequencies and cell numbers of single CD4<sup>+</sup> T cells, single CD8<sup>+</sup> T cells, double-positive, and double-negative T cells in thymus and found no significant differences in these parameters except a slight increase...
FIGURE 2 | Continued
We then compared suppression by TFR cells. Therefore, these data demonstrated maturation during TFH differentiation.

Next, we estimated late TFH differentiation at day 8 after infection in comparison to day 3, and we observed a lower abundance of SLAM+CXCR5+TFH cells differentiating from hCre-transduced SMARTA cells relative to their non-transduced compartments (Figure 3B). However, SMARTA cells transduced with empty vector expressing only GFP showed an equal frequency of TFH cells compared with non-transduced ones (Figure 3B).

Additionally, we generated Rictor<sup>−/−</sup>Cd4-Cre-SMARTA mice (called “Rictor<sup>−/−</sup>SMARTA mice” here) by crossing Rictor<sup>−/−</sup>Cd4-Cre mouse with SMARTA mice to specifically delete Rictor in SMARTA cells. Then, we adoptively transferred Rictor<sup>−/−</sup> or WT SMARTA cells (CD45.1<sup>+</sup>) into C57BL/6J (WT) (CD45.2<sup>+</sup>) and subsequently infected these recipients with LCMV Armstrong. First, we estimated early TFH differentiation at day 3 after infection, and interestingly, we found that hCre-transduced (GFP<sup>+</sup>) SMARTA cells with Rictor knocked out showed a similar frequency of Tim3<sup>+</sup>CXCR5<sup>+</sup>TFH cells to empty vector-transduced (GFP<sup>+</sup>) and non-transduced (GFP<sup>−</sup>) SMARTA cells (Figure 3A). This result suggested that early differentiated TFH cells were dispensable for TFH signaling.

in cell number of double-negative T cells in Rictor<sup>−/−</sup> mice (Figure S3A in Supplementary Material). We then compared total CD4<sup>+</sup> T cell, CD4<sup>+</sup>CD62L<sup>+</sup> T cell, and CD4<sup>+</sup>CD44<sup>+</sup> T cell populations in spleens from Rictor<sup>−/−</sup> or WT mice in the naive state and observed a slightly lower frequency but comparable cell number of total CD4<sup>+</sup> T cells in the Rictor<sup>−/−</sup> group (Figure S3B in Supplementary Material). In addition, the other two populations both displayed similar cell frequencies and numbers between Rictor<sup>−/−</sup> and WT mice (Figure S3B in Supplementary Material).

Therefore, we showed that the decrease in the TFH cell population in Rictor<sup>−/−</sup> mice was not due to defects in T cell development and homeostasis. Notably, our previous data have shown that the loss of mTORC2 signaling does not alter the generation of the T<sub>FR</sub> cells (39), which indicates that the reduction of the TFH cell population in Rictor<sup>−/−</sup> mice was probably not due to increased suppression by T<sub>FR</sub> cells. Therefore, these data demonstrated that intact mTORC2 signaling was essential for effector TFH cell differentiation in viral infection, bacterial infection, and protein immunization models.

Intact mTORC2 Signaling Is Dispensable for TFH Early Induction, but Critical for Late Maturation During TFH Differentiation

To distinguish the role of mTORC2 in early commitment induction or late maturation during TFH differentiation, respectively, we crossed Rictor<sup>−/−</sup> mice with SMARTA mice to generate Rictor<sup>−/−</sup>-SMARTA mice, which enabled us to delete Rictor in SMARTA cells by transducing the humanized -Cre (call “hCre” here) retroviral expression vector in activated SMARTA cells. To achieve this goal, we transduced activated SMARTA cells (CD45.1<sup>+</sup>) with hCre expressing vector or control empty vector and then adoptively transferred them into WT C57BL/6J recipient mice (CD45.2<sup>+</sup>), which were subsequently infected with LCMV Armstrong. First, we assessed early TFH differentiation at day 3 after infection, and interestingly, we found that hCre-transduced (GFP<sup>+</sup>) SMARTA cells with Rictor knocked out showed a similar frequency of Tim3<sup>+</sup>CXCR5<sup>+</sup>TFH cells to empty vector-transduced (GFP<sup>+</sup>) and non-transduced (GFP<sup>−</sup>) SMARTA cells (Figure 3A). This result suggested that early differentiated TFH cells were dispensable for TFH signaling.

Next, we estimated late TFH differentiation at day 8 after infection in comparison to day 3, and we observed a lower abundance of SLAM<sup>+</sup>CXCR5<sup>+</sup>TFH cells differentiating from hCre-transduced SMARTA cells relative to their non-transduced compartments (Figure 3B). However, SMARTA cells transduced with empty vector expressing only GFP showed an equal frequency of TFH cells compared with non-transduced ones (Figure 3B).

Additionally, we generated Rictor<sup>−/−</sup>Cd4-Cre-SMARTA mice (called “Rictor<sup>−/−</sup>SMARTA mice” here) by crossing Rictor<sup>−/−</sup>Cd4-Cre mouse with SMARTA mice to specifically delete Rictor in SMARTA cells. Then, we adoptively transferred Rictor<sup>−/−</sup> or WT SMARTA cells (CD45.1<sup>+</sup>) into C57BL/6J (WT) (CD45.2<sup>+</sup>) and subsequently infected these recipients with LCMV. At day 3 post-infection, we compared the expression of TCF-1 in early differentiated TFH cells from Rictor<sup>−/−</sup> and WT SMARTA cells, respectively. It turned out that TCF-1 expression level was comparable between these TFH cells, indicating that TCF-1 induction was independent of mTORC2 activity at the early TFH differentiation (Figure 3C). Taken together, these data indicated that mTORC2 signaling was not required for early induction of TFH cells, whereas it played a pivotal role in late maturation of TFH cells.

Defective Responses of mTORC2-Deficient TFH Cells Lead to Impaired Humoral Immunity

Follicular helper CD4<sup>+</sup> T cells provide help to cognate B cells to initiate GC reactions and promote the further differentiation of GC B cells into memory B cells and long-lived plasma cells (7). Given the crucial role of TFH cell in B cell responses, the defective TFH differentiation in the absence of mTORC2 appears to have a negative impact on B cell responses. To validate this hypothesis, we first compared the kinetics of GC B cells between Rictor null and WT mice at day 8, 10, and 15 after infection. As expected, we observed a great decrease in the frequencies and cell numbers of GC B cells, as defined by high expression of Fas (CD95) and peanut agglutinin (PNA) (Fas<sup>hi</sup>PNA<sup>hi</sup>) in Rictor<sup>−/−</sup> mice at all time points (Figure 4A). Likewise, we also observed similar alterations of GC B cells in the Listeria-gp66 and NP-OVA protein immunization model at day 8 after immunization (Figures
mTOR complex 2 is selectively required for late follicular helper CD4+ T (Tfh) differentiation, but not early induction. (A,B) Rictor−/− SMARTA cells were transduced with retrovirus expressing humanized-Cre (hCre) or empty vector (vector) and then transferred cells into recipients (CD45.2+) and subsequently infected with lymphocytic choriomeningitis virus (LCMV). Flow cytometry of Tfh differentiation in Rictor−/− SMARTA cells transduced (GFP+) with hCre or vector, or non-transduced (GFP−) at day 3 (A) and 8 (B) after infection (n = 4 mice per group). (C) T cell factor-1 expression in early differentiated Tfh cells at day 3 after infection. Rictor−/− or WT SMARTA (CD45.1+) cells were adoptively transferred into naive C57BL/6J (CD45.2+) mice, following intravenously infection with 2 × 10^6 plaque-forming units of LCMV and analyzed at day 3 after infection (n = 4–6 mice per group). ns, not significant, **p < 0.01 [paired two-tailed t-test (A,B), unpaired two-tailed t-test (C)]. Data are representative of two (A–C) independent experiments. Error bars are SEM (A–C).

**Figure 3** | mTOR complex 2 is selectively required for late follicular helper CD4+ T (Tfh) differentiation, but not early induction. (A,B) Rictor−/− SMARTA cells were transduced with retrovirus expressing humanized-Cre (hCre) or empty vector (vector) and then transferred cells into recipients (CD45.2+) and subsequently infected with lymphocytic choriomeningitis virus (LCMV). Flow cytometry of Tfh differentiation in Rictor−/− SMARTA cells transduced (GFP+) with hCre or vector, or non-transduced (GFP−) at day 3 (A) and 8 (B) after infection (n = 4 mice per group). (C) T cell factor-1 expression in early differentiated Tfh cells at day 3 after infection. Rictor−/− or WT SMARTA (CD45.1+) cells were adoptively transferred into naive C57BL/6J (CD45.2+) mice, following intravenously infection with 2 × 10^6 plaque-forming units of LCMV and analyzed at day 3 after infection (n = 4–6 mice per group). ns, not significant, **p < 0.01 [paired two-tailed t-test (A,B), unpaired two-tailed t-test (C)]. Data are representative of two (A–C) independent experiments. Error bars are SEM (A–C).

S4A, B in Supplementary Material). In addition to the decrease in GC B cells, GCs in spleens from Rictor−/− mice were smaller than those in WT mice, and fewer mTORC2-deficient Tfh cells could enter and localize within GCs and B cell follicles than WT control (Figure 4B).

Next, we measured LCMV-specific IgG titers in sera at multiple time points after LCMV infection and found that IgG titers were not significantly reduced from day 8 to day 32 in Rictor-deficient compared to control mice; however, they were remarkably down-regulated at day 60, indicative of impaired long-term antibody responses (Figure 4C). Consistently, GC-derived LCMV-specific ASCs in the BM were found to be greatly diminished in Rictor−/− compared with WT mice at day 60 post-infection (Figure 4D). Taken together, these data led to the notion that Rictor-deficient...
Figure 4

A

Rictor

WT

D8

D10

D15

GC B cells (%)

GC B cells (#)

B

Rictor

WT

IgD

GL-7

CD4

C

D60 post infection, bone marrow

D

Rictor

WT

ASCs in BM (#)

FIGURE 4 | Continued
mTORC2 Regulates Migratory and Functional Properties of Differentiated T<sub>FH</sub> Cells

Next, we further investigated how mTORC2 regulated T<sub>FH</sub> cell differentiation and consequently affected humoral immunity. Cognate interactions between T<sub>FH</sub> and B cells are essential for priming and maintenance of GC responses (50–52), differentiation of memory B cells and long-lived plasma cells (21, 53, 54), and complete differentiation of T<sub>FH</sub> cells (5, 9, 55). We investigated whether mTORC2 signaling was responsible for the formation of T cell–B cell junctions. To achieve this goal, we constituted SMARTA chimeras by adoptively transferring Rictor<sup>fl/fl</sup>CD4<sup>+</sup>-Cre-SMARTA (called ”Rictor<sup>−/−</sup> SMARTA” here) or WT SMARTA cells (CD45.1<sup>+</sup>) into C57BL/6J mice (CD45.2<sup>+</sup>) and subsequently infecting these recipients with LCMV. At day 8 after infection, SMARTA T<sub>FH</sub> cells were sorted from Rictor<sup>−/−</sup>- and WT SMARTA chimeras and cultured with LPS-activated B cells pulsed or not with gp66 peptide. The T cell–B cell conjugates, which were identified as CD4<sup>+</sup>B220<sup>+</sup> doublets, were quantified by flow cytometry. We found that the frequency of T cell–B cell conjugates was substantially higher in gp66-pulsed groups than unpulsed negative controls (Figure 5A). Of note, a similar frequency of T cell–B cell conjugates was observed between the Rictor<sup>−/−</sup> and WT group in the presence of gp66 (Figure 5A), and similar results were observed using gp66 tetramer<sup>+</sup> T<sub>FH</sub> cells sorted from Rictor<sup>−/−</sup>- and WT mice (Figure S5A in Supplementary Material). These data indicated that the cell adhesion between T<sub>FH</sub> and B cells was independent of mTORC2 signaling.

High expression of CXCR5 in T<sub>FH</sub> cells facilitates the response of these cells to the chemokine CXCL13 and migration toward B cell follicles (1–6), where they can engage cognate B cells. Next, to determine whether mTORC2 regulates T<sub>FH</sub> cell responses by altering its migratory pattern, we conducted a transwell migration assay to analyze the mobility of the T<sub>FH</sub> cells and ability to directionally respond to CXCL13. We added equal numbers of WT and Rictor<sup>−/−</sup> T<sub>FH</sub> cells in the upper chamber of the transwell plate and added medium supplemented with or without CXCL13 in the lower chamber. After 3 h of migration, we found that the number of migrated Rictor<sup>−/−</sup> T<sub>FH</sub> cells was largely decreased compared with the WT controls (Figure 5B), suggesting an impairment of the migratory potential of T<sub>FH</sub> cells lacking mTORC2 signaling in response to CXCL13, which might result in reduced colocalization of T<sub>FH</sub> and GC B cells and, therefore, constrained T<sub>FH</sub> differentiation and GC responses.

Finally, we assessed the impact of mTORC2 signaling on the capacity of T<sub>FH</sub> cells to provide the helper signals for B cell survival and activation. To evaluate the helper function of T<sub>FH</sub> cell in vivo, we sorted fully differentiated T<sub>FH</sub> cells from day 8-infected Rictor<sup>−/−</sup>- or WT mice (CD45.1<sup>+</sup>) and transferred equal number of these cells into Sh2d1a<sup>−/−</sup> (called Sap<sup>−/−</sup> here) (CD45.2<sup>+</sup>) recipients at day 1 of infection (Figure 5C). At day 7 after infection of recipients (6 days after cell transfer), we observed a distinct Fas<sup>+</sup>P<sub>NA</sub><sup>hi</sup> GC B cell population in mice that received exogenous T<sub>FH</sub> cells from Rictor<sup>−/−</sup>- or WT donor, while mice without cell transfer displayed barely detectable GC B cells (Figure 5D). Additionally, the number of transferred T<sub>FH</sub> cells was minimally altered (Figure 5D). Importantly, we found that mice that received Rictor<sup>−/−</sup> T<sub>FH</sub> cells exhibited an approximately 2-fold lower frequency and number of GC B cells than control mice (Figure 5D), which suggested a disrupted effector function of Rictor-deficient T<sub>FH</sub> cells.

To further confirm this point, we utilized another approach to assess T<sub>FH</sub> function in vitro. We sorted fully differentiated T<sub>FH</sub> cells from Rictor<sup>−/−</sup>- or WT mice and B cells from C57BL/6J mice at day 8 after infection of LCMV and then cultured T<sub>FH</sub> cells with B cells for 4 days in the presence of anti-CD3 and anti-IgM. Next, we found that the B cells cultured with WT T<sub>FH</sub> cells formed a distinct population of germinal center-like B cells determined by high expression of IgG1 and GL-7 (IgG1<sup>hi</sup>GL-7<sup>hi</sup>) or IgG2a and GL-7 (IgG2a<sup>hi</sup>GL-7<sup>hi</sup>), whereas B cells cultured with Rictor<sup>−/−</sup> T<sub>FH</sub> cells exhibited a much lower frequency and cell number of activated B cells than that in the WT group (Figure 5E). Moreover, the numbers of Rictor<sup>−/−</sup>- and WT T<sub>FH</sub> cells were comparable after 4 days of culture (Figure 5E). These data suggested that mTORC2 signaling was essential for the effector function of already differentiated T<sub>FH</sub> cells to promote B cell differentiation. Therefore, activated B cell responses critically depended on the presence of cognate T<sub>FH</sub> cells with competent mTORC2 signaling.

mTORC2 Plays an Essential Role in T<sub>FH</sub> Cell Lineage Identity

To understand the molecular mechanisms by which mTORC2 transcriptionally regulates T<sub>FH</sub> differentiation and function, we sorted T<sub>FH</sub> and T<sub>Eff</sub> cells from Rictor<sup>−/−</sup>- and WT mice at day 8 after infection and performed gene expression microarray analysis. Microarray analysis showed that 561 genes were upregulated and 201 genes were downregulated in Rictor-deficient T<sub>FH</sub> cells relative to their WT counterparts (Table S3 in Supplementary Material, Hao et al.).

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FIGURE 5 | Continued
To further analyze the transcriptomic alterations in Rictor−/− TFH cells, we next selected sets of genes from published datasets (GEO accession codes GSE21379 and GSE21381) that are upregulated and downregulated in TFH cells compared with non-TFH cells (56), for GSEA. GSEA analysis illustrated that the genes related to the T FH cell signature (upregulated in T FH cells) were more enriched in WT T FH cells, but not Rictor−/− T FH cells (Figure 6A). By contrast, Rictor−/− T FH cells showed enrichment for the gene set associated with the non-T FH lineage (downregulated in T FH cells) (Figure 6A). We then assessed 71 genes from the GSEA results and observed a distinct gene expression profile between Rictor−/− T FH cells and WT T FH cells (Figure 6B). These results suggested that mTORC2 signaling deficiency resulted in disruption of T FH lineage specification.

Next, we selected several genes that are closely involved in T FH differentiation and function from the microarray results and confirmed their alterations by quantitative polymerase chain reaction (qPCR) (Figure 6C). We noted that the expression of Bcl6, Ascl2, and Tsf7, which encode key transcription factors in T FH cells, were remarkably downregulated in Rictor-deficient T FH compared with WT T FH cells (Figure 6C). Moreover, we also found that Rictor−/− T FH cells exhibited a lower mRNA abundance of Maf, a c-Maf encoding gene, which induces the expression of IL-21 in T FH cells to support GC development (57, 58) (Figure 6C). Accordingly, both the levels of Il21 and Il21r were downregulated in Rictor-null T FH compared with WT cells (Figure 6C). Similarly, the expression levels of Cd40lg and Il4, encoding CD40L and IL-4 to promote B cell differentiation, were lower in Rictor−/− T FH cells (Figure 6C). Additionally, the expression levels of both Il6ra and Il6st, which encode the IL-6R and gp130 receptors for IL-6, respectively, and are essential for instructing early T FH differentiation, were decreased in Rictor−/− T FH cells (13, 59) (Figure 6C). In addition, we observed a reduction of Cxcr5 and Pdcd1, which respectively encode the T FH distinguishing markers CXCR5 and PD-1 in Rictor−/− T FH cells (Figure 6C). These data suggested that mTORC2 played a critical role in maintaining both T FH lineage identity and functionality.

**DISCUSSION**

In this study, we focused on dissecting the role of mTORC2 in the temporal regulation of T FH differentiation and effector function upon viral infection. mTORC1 responds to diverse environmental cues, including amino acids, stress, oxygen, energy, and growth factors (25), while mTORC2 is insensitive to nutrients but activated by growth factors (60); however, knowledge is scarce regarding mTORC2 stimuli compared with mTORC1. In our study, we demonstrated that the combination of ICOS and CD3 acted as upstream activators of mTORC2 signaling in T FH cells. Moreover, we found that T FH cells exhibited higher mTORC2 activity than Th1 cells upon stimulation, suggesting that T FH cells might be dependent on mTORC2 activity to a greater degree. Accordingly, the generation of T FH cells, not Th1 cells, was specifically impaired after deletion of Rictor, implying that T FH cells were more sensitive to reduced mTORC2 activity. Furthermore, we found that mTORC2 was required for full differentiation of T FH cells in the B cell-dependent phase, but not priming. In addition to T FH generation, mTORC2 was also necessary for T FH effector functions for helping B cells. Therefore, both lower total numbers and defective functions of T FH cells synergistically resulted in aberrant humoral immunity, characterized by decreased GC B cells, a smaller GC size, poor production of virus-specific IgG, and diminished ASCs in BM. These data collectively indicated a crucial role of mTORC2 signaling in the full commitment of T FH cells and humoral immunity.

Two groups recently reported that mTORC2 signaling regulates T FH differentiation and GC responses in the mesenteric lymph nodes and PPs under the steady state as well as upon protein immunization via distinct mechanistic (41, 42). Yang and colleagues found that mTORC2 promoted T FH cell survival but did not affect proliferation through the phosphorylation of AKT (42). In addition, they showed that the mTORC2–AKT–TCF-1 axis was important for T FH differentiation (42). Another group showed that ICOS-mTORC2-Foxo1 signaling axis was required for T FH differentiation by promoting glucose metabolism and the T FH transcriptional program (41). Moreover, they observed a reduction of T FH and GC B cells after abrogation of mTORC2 signaling in the LCMV acute infection model, which was repeatable in our study (41). However, these studies did not dissect the different roles of mTORC2 in early and late stages during T FH differentiation, respectively, nor assess the influence of mTORC2 on T FH effector functions. Here, we provided unambiguous evidence showing that mTORC2 signaling was required for the late, but early priming, stage of T FH differentiation; in addition, mTORC2 was important for supporting T FH effector functions.

The complete differentiation and maintenance of T FH cells depends on the necessary signals, including CD80, CD86, ICOSL, and CD40, provided by cognate B cells (61–64). To receive signals from B cells, it is critical for T FH cell to migrate...
FIGURE 6 | Continued
to B cell follicles and colocalize with B cells after priming, and subsequently to form T cell–B cell junctions. Although we found that T cell–B cell adhesion was not affected by mTORC2, the T<sub>FH</sub> migratory capacity in responses to CXCL13 was impaired in the absence of mTORC2, which resulted from downregulated CXCR5 expression. Furthermore, it has been reported that mTORC2 regulates cytoskeletal remodeling and cell migration by phosphorylating a series of proteins of PKC family, including PKC-α, PKC-δ, PKC-ζ, PKC-γ, and PKC-ε (65–69). Therefore, in addition to downregulated CXCR5 expression, mTORC2 may play a potential role in modulating T<sub>FH</sub> cell migration via the mTORC2–PKC axis; however, the underlying mechanisms remain unclear and merit further exploration. Taken together, a compartmental segregation between T<sub>FH</sub> and B cells formed after abrogation of mTORC2 signaling, preventing T<sub>FH</sub> full differentiation in the B cell-dependent phase: fewer T<sub>FH</sub> cells were maintained, underwent further development into GC T<sub>FH</sub> cells, and accomplished functional maturation. Accordingly, we found that mTORC2-deficient T<sub>FH</sub> cells failed to provide adequate help to B cells, even under in vitro culture conditions, which eliminated the interferences from reduced colocalization of T<sub>FH</sub> and B cell. This indicated that mTORC2 signaling was critical for T<sub>FH</sub> effector functions assisting B cells, at least in part because mTORC2 promoted the expression of T<sub>FH</sub> function-related genes, such as Il4, Il21, and Cd40lg.

Our study results led to the conclusion that mTORC2 was involved in the regulation of T<sub>FH</sub> cell late differentiation and effector functions. Moreover, it is well accepted that exaggerated T<sub>FH</sub> responses and functions provide a great contribution to the pathogenesis of autoimmune diseases characterized by spontaneous GC formation and autoantibody production, such as systemic lupus erythematosus and rheumatoid arthritis (70–72). Therefore, suppression of T<sub>FH</sub> responses by targeting the mTORC2 signaling pathway might serve as a potential therapeutic strategy for autoimmune diseases.

**ETHICS STATEMENT**

All mouse experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of the Third Military Medical University.

**AUTHOR CONTRIBUTIONS**

YH, ZY, YZW, and LY designed and oversaw experiments. YH, YFW, XL, XY, PW, QT, XC, ZL, JW, ZX, XZ, and YZ performed experiments. YH and QB analyzed experiments. YH and LY wrote the paper.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01127/full#supplementary-material.
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