mTORC1 and mTORC2 Kinase Signaling and Glucose Metabolism Drive Follicular Helper T Cell Differentiation

Highlights

- Raptor or Rictor deletion impairs GC and Tfh responses and IgA production
- mTORC2 but not mTORC1 signals via Foxo1 inhibition to drive Tfh cell differentiation
- mTORC1 and mTORC2 promote foreign antigen and LCMV-induced GC and Tfh responses
- mTOR links ICOS and Glut1 to anabolic metabolism for Tfh cell differentiation

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In Brief

Follicular helper T (Tfh) cells are essential for efficient humoral immune responses. Zeng et al. identify mTOR signaling and glucose metabolism as critical regulators of Tfh cell differentiation in response to foreign antigen challenge and at steady state in Peyer’s patches.

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mTORC1 and mTORC2 Kinase Signaling and Glucose Metabolism Drive Follicular Helper T Cell Differentiation

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SUMMARY

Follicular helper T (Tfh) cells are crucial for germinal center (GC) formation and humoral adaptive immunity. Mechanisms underlying Tfh cell differentiation in peripheral and mucosal lymphoid organs are incompletely understood. We report here that mTOR kinase complexes 1 and 2 (mTORC1 and mTORC2) are essential for Tfh cell differentiation and GC reaction under steady state and after antigen immunization and viral infection. Loss of mTORC1 and mTORC2 in T cells exerted distinct effects on Tfh cell signature gene expression, whereas increased mTOR activity promoted Tfh responses. Deficiency of mTORC2 impaired CD4+ T cell accumulation and immunoglobulin A production and aberrantly induced the transcription factor Foxo1. Mechanistically, the costimulatory molecule ICOS activated mTORC1 and mTORC2 to drive glycolysis and lipogenesis, and glucose transporter 1-mediated glucose metabolism promoted Tfh cell responses. Altogether, mTOR acts as a central node in Tfh cells by linking immune signals to anabolic metabolism and transcriptional activity.

INTRODUCTION

Follicular helper T (Tfh) cells are specialized effector T cells that stimulate B cells in germinal center (GC) follicles to produce long-lived high-affinity immunoglobulins (Crotty, 2014). Although the GC response is usually induced by foreign antigen stimulation in peripheral lymphoid organs, it is continuously present in Peyer’s patches (PPs) and contributes to the production of secretive immunoglobulin A (IgA) for gut immune homeostasis. This spontaneous GC formation is maintained by perpetual exposure to gut microbiota and strictly depends upon Tfh cell help (Fagarasan et al., 2010). GC formation and Tfh cell development in PPs are abrogated in ICOS-deficient mice (Gigoux et al., 2009; Iiyama et al., 2003), illustrating the essential role of ICOS in both peripheral and mucosal Tfh cell differentiation. However, signaling mechanisms underlying Tfh cell differentiation, particularly in PPs, remain essentially unexplored.

T cell differentiation is accompanied by dynamic metabolic reprogramming (Maclver et al., 2013). One of the crucial regulators of anabolic metabolism is mechanistic target of rapamycin (mTOR) signaling, which is composed of two distinct kinase complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2) that are characterized by the signature components Raptor and Rictor, respectively (Chi, 2012). Recent studies have revealed discrete functions of mTORC1 and mTORC2 in effector CD4+ T cell differentiation (Chi, 2012). The metabolic function of mTOR signaling in T cells has been mainly ascribed to mTORC1 (Finlay et al., 2012; Yang et al., 2013; Zeng et al., 2013), while mTORC2 negatively controls anabolic metabolism in CD8+ T cells (Pollizzi et al., 2015). A recent study shows that shRNA-mediated silencing of mTOR or Raptor, but not Rictor, in activated T cells enhances Tfh cell differentiation (Ray et al., 2015), indicating an inhibitory role of mTORC1 and metabolism in Tfh cell differentiation. However, this conclusion appears to be contrary to the positive roles of PI3K signaling in Tfh cell responses (Gigoux et al., 2009; Rolf et al., 2010). Furthermore, Bcl6 has been shown to directly inhibit glycolytic genes in T helper 1 (Th1) and CD8+ T cells (Oestreich et al., 2014). Yet, Bcl6+ Tfh cells show higher proliferation and increased expression of cell-cycle-related genes compared to Bcl6- Tfh cells (Kitano et al., 2011), and Tfh cells also exhibit a higher rate of proliferation...
than other effector T cells (Lüthje et al., 2012). Finally, expression of the glucose transporter Glut1 and increased glycolysis have been shown to promote effector T cell function and proliferation (Jacobs et al., 2008; Macintyre et al., 2014). Detailed metabolic requirements and the interplay with signaling events in Thf cells, however, remain to be established.

Here we tested the role of mTORC1 and mTORC2 and glucose metabolism in Thf cell differentiation and the ability to promote GC responses. We found that both mTORC1 and mTORC2 were crucial positive determinants of Thf and GC responses by orchestrating discrete transcriptional programs. mTORC1 and mTORC2 were activated by ICOS to promote metabolism including glycolysis and lipogenesis, while mTORC1 also directly contributed to ICOS expression. Importantly, direct manipulation of metabolic activities through the transcription factor Myc or Glut1 modulated Thf cell responses. Our study demonstrates that mTORC1 and mTORC2 play non-redundant roles in Thf cell differentiation by linking immune signals to anabolic metabolism and transcriptional activity.

RESULTS

mTOR Signaling Is Sufficient and Necessary for GC and Thf Responses in PPs

To investigate how mTOR signaling shapes GC and Thf cell responses under steady state, we examined PPs from Cd4crePtenfl/fl mice with T cell-specific deletion of PTEN, a crucial negative regulator of PI3K and mTOR signaling (Chi, 2012). PPs from wild-type (WT) mice contained a sizable population of B cells expressing GC markers GL-7 and Fas, and of T cells expressing Thf cell markers PD-1 and CXCR5 (Figures 1A and 1B). PTEN deficiency significantly increased the frequencies of GC B cells and Thf cells in PPs (Figures 1A and 1B). Because PTEN also possesses functions independent of PI3K-Akt (Shen et al., 2007; Song et al., 2011), we used Cd4Cre to ectopically express a constitutively active form of the PI3K catalytic subunit p110α (encoded by Pik3ca) (Srinivasan et al., 2009) in T cells (referred to as Pik3ca+). GC B cell and Thf cell frequencies were markedly enhanced in Cd4crePik3ca+ mice (Figures S1A and S1B). Next, to test the requirement of mTOR, we treated WT mice with rapamycin, an immunosuppressant that targets mTORC1 primarily but also mTORC2 under long-term treatment (Chi, 2012). Rapamycin strongly reduced GC B and Thf cells in PPs (Figure 1C). Therefore, over-activation of PI3K-mTOR and inhibition of mTOR exert positive and negative effects on GC B and Thf cells in PPs, respectively.

Although PI3K-Akt is a classical activator of mTOR (Chi, 2012), PI3K-Akt-independent pathways of mTOR activation have been identified (Deane et al., 2007; Donahue and Fruman, 2007; Finlay et al., 2012). To establish the roles of mTOR and to dissect the two mTOR complexes in this process, we generated Cd4creRaptorfl/fl and Cd4creRictorfl/fl mice and examined T cell homeostasis and GC responses in PPs. Compared with splenic T cells, T cells from PPs contained a considerably higher frequency of CD44hiCD62Llo memory-phenotype (MP) T cells (Figure S1C). In PPs, Rictor deficiency resulted in a more pronounced reduction of MP T cells than Raptor deficiency (Figure S1D), suggesting a preferential role for mTORC2 in PPs. Indeed, we observed a reduced frequency and number of CD4+ T cells in PPs from Cd4creRictorfl/fl mice, but not from Cd4creRaptorfl/fl mice (Figure 1D and Figure S1E).

We next examined GC B cells and Thf cells, and found that the frequencies of these populations were significantly reduced in Cd4creRictorfl/fl or Cd4creRictorfl/fl mice (Figures 1E and 1F). Therefore, both mTORC1 and mTORC2 are required for Thf cell generation in PPs, with mTORC2 making an additional contribution to T cell accumulation in PPs.

A unique feature of B cell responses in GCs of PPs is the switching of B cell isotype from IgM to IgA to maintain intestinal homeostasis (Fagarasan et al., 2010). Staining of surface IgA showed significantly reduced IgA expression on B cells from Cd4creRictorfl/fl mice (Figure 1G). Moreover, Cd4creRictorfl/fl mice had reduced IgA concentration in fecal extracts (Figure 1H). B cell IgA expression and fecal IgA production were trended lower in Cd4creRaptorfl/fl than WT mice, but the changes were smaller or did not reach statistical significance (Figures 1G and 1H). Thus, mTORC2 plays a prominent role in IgA production in PPs.

T follicular regulatory T cells (Tfr) constrain Thf cell differentiation (Chung et al., 2011; Linterman et al., 2011), and so the diminished Tfh cells in PPs in Raptor or Rictor-deficient mice could be due to elevated Tfr cell function. However, Raptor deficiency in Treg cells abrogates their suppressive function (Zeng et al., 2013) and is unlikely to account for the diminished Tfh cells in Cd4creRictorfl/fl mice. To examine whether mTORC2 in Treg cells could affect Tfh cell differentiation, we analyzed Cd4creRictorfl/fl mice and observed normal Tfr cells in PPs (Figure S1F). Also, Treg-specific deletion of Rictor via Foxp3-Cre had little effect on Tfh and GC B cells in PPs (Figure S1G). The blunted Tfh cell differentiation in Raptor or Rictor-deficient mice is thus unlikely to arise from Tfr cell functional enhancement.

mTOR in Activated T Cells Is Critical for Tfh Cell Differentiation and GC Formation in PPs

Because mTORC1 is required for early T cell activation (Yang et al., 2013), compromised T cell activation in the Cd4-Cre-mediated gene deletion could complicate data interpretation. We therefore used a deleter line with Cre expression mediated by the OX40 promoter to induce deletion in CD4+ T cells after initial activation (Kang et al., 2013). As expected, Raptor and Rictor mRNA were not completely depleted in CD4+ T cells until 3 days after in vitro stimulation (Figure 2A). Also, CD4+ T cells from Ox40creRaptorfl/fl and Ox40creRictorfl/fl mice had largely normal proliferation within the first 2 days of stimulation (Figure 2B).

MP T cells are known to express OX40 (Klinger et al., 2009). In Ox40cre mice crossed with a Cre recombination reporter, efficient deletion of the reporter allele was observed in MP but not naive CD4 T cells in PPs (Figure S2A). PPs from Ox40creRictorfl/fl but not Ox40creRaptorfl/fl mice contained a smaller population of CD4+ MP T cells than their WT counterparts (Figure S2B). Confocal microscopic examination of PPs from Ox40creRictorfl/fl and Ox40creRictorfl/fl mice revealed highly reduced staining with peanut agglutinin (PNA), a specific dye for GCs (Figure 2C). Flow cytometry analysis verified markedly reduced GC B and Thf cells in PPs from these mice (Figures 2D and 2E). In contrast, T cell proliferation and apoptosis (data not shown) and generation of Th1, Th2, and Th17 cells (Figure S2C) were largely unaffected.
To determine whether these Tfh cell defects are cell intrinsic, we constructed mixed bone marrow (BM) chimeras by reconstituting lethally irradiated TCR-β and TCR-δ double-deficient (Tcrβ−/−Tcrδ−/−) mice with BM cells from CD45.1+ mice, together with those from WT and Cd4crePtenfl/fl mice. Right shows the frequency of GC B cells or Tfh cells.

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Figure 2. Deletion of Raptor and Rictor via OX40-Cre Impairs Tfh Cell Differentiation and Immune Homeostasis in PPs

(A) RNA analysis of Rptor and Rictor in freshly isolated or activated T cells from WT, OX40\textsuperscript{Rptor}\textsuperscript{fl/fl}, or OX40\textsuperscript{Rictor}\textsuperscript{fl/fl} mice.

(B) Naive T cells from WT, OX40\textsuperscript{Rptor}\textsuperscript{fl/fl}, and OX40\textsuperscript{Rictor}\textsuperscript{fl/fl} mice were stimulated with anti-CD3 alone or anti-CD3 plus anti-CD28 for different days, followed by [\textsuperscript{3}H]-thymidine incorporation analysis.

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mTORC2 Signaling Promotes Tfh Cell Responses through Inhibition of Foxo1

We examined the role of mTORC2 in the regulation of molecules involved in T cell homeostasis. CD4+ T cells in PPs of OX40cre/Rictorfl/fl mice exhibited increased CD62L, CD127 (IL-7Rα), and CCR7 and reduced CD69 expression (Figure 3A). These molecules are known to be under the control of Foxo1 (Kerdiles et al., 2009; Ouyang et al., 2009). Considering the requirement of Rictor in CD4+ T cell abundance in PPs selectively (Figure 2G), we asked whether T cells from different lymphoid tissues exhibit differential expression of Foxo1-dependent targets. MP CD4+ T cells from PPs showed highest expression of CD69, but lowest expression of CD127, CCR7, and CD62L (Figure 3B), as well as S1pr1 and Kll2, also targets of Foxo1 (Figure 3C). Thus, MP T cells from PPs have reduced Foxo1-dependent gene expression compared to those from peripheral lymphoid organs.

Foxo1 is a key negative regulator for Tfh cell differentiation (Stone et al., 2015; Xiao et al., 2014). Akt phosphorylates Foxo proteins, leading to their nuclear export and degradation (He-drick et al., 2012), but it remains unknown whether Foxo proteins functionally interplay with mTOR in CD4+ T cells. We therefore examined subcellular localization of Foxo1 in activated T cells from OX40cre/Raptorfl/fl and OX40cre/Rictorfl/fl mice. Anti-ICOS stimulation triggered robust Foxo1 nuclear export in WT and Raptor-deficient T cells, but not in Rictor-deficient T cells (Figure 3D). These results indicate that ICOS affects Foxo1 activity in T cells through mTORC2.

We next determined whether ablation of Foxo1 could restore defective Tfh cells in Rictor-deficient mice. Due to development of autoimmunity in OX40cre/Foxo1fl/fl mice (probably due to Treg cell defects [Ouyang et al., 2012]), we used the Cd4cre system to generate compound mutant mice. Inactivation of Foxo1 in T cells largely restored Tfh and GC B cells in Cd4cre/Rictorfl/fl; Foxo1fl/fl mice (Figures 3A and 3B). Given the disrupted T cell homeostasis in Cd4cre/Foxo1fl/fl mice (Kerdiles et al., 2009; Kerdiles et al., 2010; Ouyang et al., 2009; Ouyang et al., 2010), we also examined whether a partial loss of Foxo1 could ameliorate the Tfh defect. The reduced Tfh and GC cell responses in Cd4cre/Rictorfl/fl; Foxo1fl/fl mice were significantly restored in Cd4cre/Rictorfl/fl; Foxo1f1/4 mice, albeit to a partial degree (Figures 3E and 3F). Therefore, Foxo1 activity was upregulated in the absence of mTORC2, and reduction of Foxo1 activity partially restored defective Tfh cells.

mTORC1 and mTORC2 Orchestrate Overlapping and Discrete Gene-Expression Programs

To further explore how mTORC1 and mTORC2 control T cell homeostasis and Tfh cell differentiation, we compared gene-expression profiles of MP T cells from PPs of Cd4cre/Raptorfl/fl and Cd4cre/Rictorfl/fl mice (Cd4-Cre was used to ensure efficient deletion). The differentially expressed genes from Raptor or Rictor-deficient T cells (with greater than 0.5 log2 fold change compared to WT) corresponded to 1,802 probes. Among them, 209 probes (R1) were concordantly changed, while 45 probes (R3) showed opposite direction of changes between the two mutant cells (Figure 3G). More probes, however, showed altered expression in only one of the mutant cells, with 1,093 probes in Raptor-deficient (R4) and 455 probes in Rictor-deficient cells (R2) (Figure 3G). A heatmap of differentially expressed genes showed that Raptor and Rictor control both overlapping and distinct gene-expression profiles (Figure S3C).

We performed gene-set enrichment analysis (GSEA) to compare gene expression of Raptor or Rictor-deficient T cells versus WT cells. This unbiased approach identified a number of downregulated pathways in both Raptor and Rictor-deficient T cells, including cell cycle, pyruvate metabolism and citric acid TCA cycle, glucose metabolism, and lipid metabolism (Figure S3D). In contrast, cholesterol biosynthesis was suppressed in Raptor-deficient cells selectively, while cytokine-cytokine receptor interaction and chemokine-chemokine receptor pathways were upregulated in the absence of Rictor, but not Raptor (Figure S3D). When applying GSEA to curate the transcriptional signatures of Tfh cells (Choi et al., 2015; Yusuf et al., 2010), we found inhibition of Tfh cell gene signatures in Raptor and Rictor-deficient cells (Figure 3H). There was little overlap between Raptor and Rictor-dependent Tfh cell signatures, suggesting that mTORC1 and mTORC2 mediate discrete programs of Tfh cell differentiation (Figure 3H). This was further supported by ingenuity pathway analysis (IPA) that revealed activation of immune cell trafficking in the absence of Rictor but not Raptor (Figure S3E). Thus mTORC1 and mTORC2 orchestrate both overlapping and distinct gene-expression programs in cell metabolism, immune functions, and Tfh cell differentiation.

mTORC1 and mTORC2 Are Essential for Tfh Cell Induction upon LCMV Infection

We next explored the roles of mTOR in Tfh cell differentiation in peripheral immune tissues. Under steady state, the spleen from WT, OX40cre/Raptorfl/fl, or OX40cre/Rictorfl/fl mice showed no differences in GC B cells (Figure S4A) or Tfh cells (Figure S4B). To examine GC reaction in response to acute viral infection, we challenged mice with LCMV Armstrong strain. At day 8 after infection, GC B cells were reduced in both OX40cre/Raptorfl/fl and OX40cre/Rictorfl/fl mice (Figures 4A and 4B). Furthermore, IgD+CD138+ plasma B cells were significantly reduced in OX40cre/Raptorfl/fl mice and, to a lesser degree, OX40cre/Rictorfl/fl mice.
mice (Figures 4C and 4D). Examination of Tfh cell markers PD-1 and CXCR5 (Figures 4E and 4F) or Bcl6 and CXCR5 (Figures S4C and S4D) revealed a significant reduction of Tfh cells in both OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> and OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice. To investigate whether these defects were cell-intrinsic, we constructed mixed BM chimeras by reconstituting sub-lethally irradiated Rag<sup>1<sup>−/−</sup></sup> mice with BM cells from CD45.1<sup>−/−</sup> mice, together with those from WT, OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> or OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice. At 2 months after reconstitution, we infected chimeras with LCMV. While T cells derived from CD45.1<sup>−/−</sup> BM cells had normal Tfh cell differentiation (Figures 4G and 4H, right panels), T cells from OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> or OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> BM cells had reduced Tfh cells relative to WT cells (Figures 4G and 4H, left panels).

Given the shared developmental history between Tfh and Th1 cells (Nakayama et al., 2011), we investigated LCMV-induced Th1 cell differentiation. Raptor deficiency reduced Th1 cells (T-bet/CXCR5<sup>+</sup>) (Figure S4E), while Rictor deficiency slightly elevated Th1 cells (Figure S4F). Therefore, both mTORC1 and mTORC2 are required for Tfh and GC B cell responses upon viral infection, whereas mTORC1 but not mTORC2 contributes to Th1 cell differentiation.

**mTORC1 and mTORC2 Promote Foreign Antigen-Induced Tfh Cell Responses**

To further assess the role of mTORC1 and mTORC2 in GC responses, we immunized mice with ovalbumin linked to 4-hydroxy-3-nitrophenylacetyl hapten (NP-OVA) (Shrestha et al., 2015). Both OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> and OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice had reduced GC B cells in the spleen (Figures 5A and 5B) and draining lymph nodes (data not shown). Confocal microscopic analysis revealed smaller size of GCs (Figure 5C), and analysis of serum antibodies showed reduced production of anti-NP IgM and IgG, and IgG subtypes IgG1, IgG2c, and IgG3 titers (Figure 5D). OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> and OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice also had markedly reduced Tfh cells, as revealed by co-expressed markers PD-1 and CXCR5 (Figures 5E and 5F), ICOS and CXCR5 (Figure S5A), or Bcl6 and CXCR5 (Figure S5B). Altogether, mTORC1 and mTORC2 are required for Tfh cell differentiation, GC formation, and humoral responses after foreign antigen immunization.

To test whether the requirements of mTORC1 and mTORC2 in Tfh cell differentiation are cell-autonomous, we immunized mixed BM chimeras (as described in Figures 4G and 4H) with NP-OVA.

T cells derived from OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> or OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> BM cells had diminished Tfh cell generation (Figures 5G and 5H). Moreover, to exclude altered TCR repertoire as a potential contributing factor, we bred OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> and OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice with OT-II transgenic mice expressing OVA-specific TCRs. We transferred naive T cells from these mice into CD45.1<sup>−/−</sup> recipients and immunized them with NP-OVA. Compared with WT OT-II T cells, those deficient in Raptor or Rictor generated significantly reduced Tfh cells (Figure 5I). Collectively, mTORC1 and mTORC2 are required for Tfh cell differentiation after immunization via T cell-intrinsic and antigen-specific mechanisms.

**Raptor or Rictor Deficiency Impairs ICOS-Induced Signaling and Metabolism**

We determined upstream and downstream pathways mediated by mTORC1 and mTORC2 for Tfh cell differentiation. Expression of Tfh cell-associated transcription factors Bcl6 and IRF4 (Bollig et al., 2012) was undisturbed by Raptor or Rictor deficiency (Figure 6A). ICOS, which regulates late-stage Tfh cell differentiation via Foxo1 (Stone et al., 2015; Weber et al., 2015), was modestly reduced in the absence of Raptor, but not Rictor, in the spleen after immunization or PPs under steady state (Figure 6B). Because mTORC2 mediates ICOS-induced Foxo1 nuclear exclusion (Figure 3D), we examined expression of Foxo1-dependent target genes in splenic Tfh cells after NP-OVA immunization. Rictor-deficient Tfh cells expressed higher amounts of Foxo1 target genes, including Klf2, Il7r, and Ccr7 (Figure 6C). Flow cytometry analysis revealed increased expression of CD26L and CD127 and reduced expression of CD69 (Figure 6D). Altogether, loss of Raptor modestly reduces ICOS expression, whereas Rictor deficiency upregulates Foxo1 gene targets.

To examine ICOS-induced signaling events, we stimulated T cell blasts from OX40<sup>Cre/Rptor<sup>fl/fl</sup></sup> and OX40<sup>Cre/Rictor<sup>fl/fl</sup></sup> mice with anti-ICOS, which induced phosphorylation of S6 and Akt (serine 473) that are the respective targets of mTORC1 and mTORC2. These phosphorylation events were profoundly lost in the absence of Raptor and Rictor, respectively (Figure 6E). Compared to anti-CD3 stimulation alone, anti-CD3 plus anti-ICOS promoted cell lipogenesis (Figures 6F and 6G) and glycolysis (Figure 6H), but such enhancement was blunted by deficiency of Raptor or Rictor (Figures 6F–6H). Moreover, defective glucose uptake was evident in vivo in Tfh cells from immunized...
OX40<sup>cre</sup>R<sup>ptor<sup>fl/fl</sup> and OX40<sup>cre</sup>R<sup>ictor<sup>fl/fl mice, as indicated by staining with the glucose analog, 2-((N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Figure 6 I). Finally, we transferred naive OT-II T cells to recipient mice, and NP-OVA immunization activated all donor T cells by day 5, as indicated by the uniformly high CD44 expression (Figure S6A). We compared Tfh cells to non-Tfh cells (Figure S6 B) in terms of cell size, 2-NBDG staining, CD71 and CD98 expression (Figure S6 C), and expression of key glycolytic genes (Figure S6 D). In all cases, Tfh cells exhibited higher metabolic activities. Therefore, mTORC1 and mTORC2 link ICOS stimulation to metabolic activities.

The proto-oncogene Myc mediates metabolic reprogramming of T cells responding to antigen stimulation (Wang et al., 2011). To test whether Myc was important for Tfh cell differentiation, we generated OX40<sup>cre</sup>Myc<sup>fl/fl</sup> mice. OX40<sup>cre</sup>Myc<sup>fl/fl</sup> mice showed a profound reduction of GC and Tfh cells following antigen stimulation (Figure 6 J), as well as in PPs under steady state (Figure 6K). Thus, Myc function is required for Tfh cell differentiation.

Glucose Metabolism Regulates Tfh Cell Differentiation

We next directly tested the metabolic requirements of Tfh cells. To this end, we differentiated naive CD4<sup>T</sup> cells in vitro to...
Figure 5. OX40<sup>creRptor<sup>fl/fl</sup> and OX40<sup>creRictor<sup>fl/fl</sup> Mice Fail to Mount Efficient GC Reaction after Immunization via Cell-Intrinsic and Antigen-Specific Mechanisms

(A and B) Flow cytometry of GC B cells in spleen from WT and OX40<sup>creRptor<sup>fl/fl</sup> mice (A) or OX40<sup>creRictor<sup>fl/fl</sup> mice (B) at 7 days after intraperitoneal immunization of mice with NP-OVA plus LPS in alum. Right shows the frequency and number of GC B cells. **p < 0.01, ***p < 0.001, ****p < 0.0001 (Mann-Whitney test).

(C) Immunohistochemistry of GCs in the mesenteric lymph nodes of WT, OX40<sup>creRptor<sup>fl/fl</sup> and OX40<sup>creRictor<sup>fl/fl</sup> mice at 7 days after immunization. Scale bars show 100 μm. Right shows calculated GC size.

(D) Measurements of anti-NP immunoglobulins in serum from immunized mice, presented as absorbance at 450 nm (A<sub>450</sub>) in ELISA.

(E and F) Flow cytometry of Tfh cells in spleen from WT and OX40<sup>creRptor<sup>fl/fl</sup> mice (E) or OX40<sup>creRictor<sup>fl/fl</sup> mice (F) at 7 days after intraperitoneal immunization of mice with NP-OVA plus LPS in alum. Right shows the frequency and number of Tfh cells.

(legend continued on next page)
generate Tfh-like cells (Awe et al., 2015), which expressed IL-21, Bcl6, CXCR5, PD-1, and ICOS (Figures S7A–S7C). Metabolite profiles were generated using high-resolution mass spectrometry to compare the metabolome of Tfh-like cells with that of activated T cells (Tact) and Th1 and Th17 cells. Each subset was metabolically distinct (Figures 7A and 7B). Pathway analyses of altered metabolites showed that amino acid, amino sugar metabolism, and glycolytic intermediates trended higher in Tfh-like cells, but Tfh-like cells had lower amounts of TCA metabolites and intermediates in cysteine and glycerophospholipid metabolism (Table S1). Consistent with T cell reliance on both glucose and mitochondrial metabolism pathways, treatment of in vitro differentiated Tfh-like cells with low doses of either glycolysis inhibitor 2-deoxyglucose (2-DG) or electron transport inhibitor rotenone led to reduced number of IL-21-producing CD4+ T cells (Figure 7C).

Our data show that mTOR signaling promotes glucose metabolism and uptake in Tfh cells in vivo. Indeed, in vitro induced Tfh-like cells expressed elevated Glut1 (Figures 7D and 7E). Likewise, Glut1 expression was higher on Tfh cells relatively to other non-Tfh CD4+ T cells from PPs (Figure 7F) and spleens (Figure S7D). Glut1 expression was partly dependent on mTORC1 signaling, as rapamycin treatment reduced Glut1 expression in vitro (Figure S7E and S7F). Consistent with this finding, Glut1 expression was reduced in Raptor-deficient, but not Rictor-deficient, Tfh cells after LCMV infection (Figure S7G). Rapamycin treatment also suppressed glucose uptake (Figure S7H) of in vitro Tfh-like cells. T cells with transgenic expression of Glut1 (Slc2a1-Tg), however, were able to maintain elevated glucose uptake even when treated with rapamycin (Figure S7H). Short-term treatment with rapamycin did not affect IL-21 expression in Slc2a1-Tg T cells, while it suppressed IL-21 expression in WT T cells (Figure 7G). Long-term treatment of rapamycin reduced IL-21 expression in both WT and Slc2a1-Tg T cells, but the latter still showed increased resistance to rapamycin (Figure S7I).

The role of glucose uptake in Tfh cell differentiation was next tested by directly modulating Glut1 expression. While no difference in Tfh cell responses was observed in mice with T cell-specific deletion of Glut1 (Cd4creSlc2a1fl/fl) after immunization with sheep red blood cells (SRBC) (Figure S7J), this genetic modification did not alter glucose uptake in Tfh cells (Figure S7K) and instead led to compensatory upregulation of Glut3 (Figure S7L). Thus, we tested whether increased glucose uptake in Slc2a1-Tg mice altered Tfh cell differentiation. Relative to non-transgenic controls, GC B cells and IgA-expressing B cells were elevated in PPs from Slc2a1-Tg mice under steady state (Figures 7H and 7I). Examination of Tfh cell signature proteins, CXCR5, PD-1, ICOS, and Bcl6, revealed increased Tfh cells in the PPs of Slc2a1-Tg mice (Figure 7J and Figures S7M and S7N). Further, we immunized WT mice with KLH and analyzed Glut1 expression on Tfh cells in the draining lymph nodes. Similar to steady state, Tfh cells expressed higher Glut1 relatively to activated non-Tfh CD4+ cells (Figure S7O). Moreover, Tfh cells were significantly enriched in immunized Slc2a1-Tg mice (Figure 7K). Thus, Glut1 promotes the generation of Tfh cells under steady state and upon foreign antigen challenge.

DISCUSSION

Despite the recent identification of specific receptors and transcription factors underlying Tfh cell differentiation (Crotty, 2014), the intracellular processes linking these events remain poorly understood. Here we identified mTORC1 and mTORC2 signaling as a key mechanism that links ICOS to anabolic metabolism and transcriptional regulation, thereby driving Tfh cell differentiation and humoral immunity. mTORC1 and mTORC2 orchestrate distinct transcriptional programs, with mTORC2 selectively affecting Foxo1 activity. Moreover, ICOS stimulation strongly upregulated glycolysis and lipid biosynthesis, and loss of either mTORC1 or mTORC2 diminished such metabolic activities. These data, together with the results from Glut1-transgenic and Myc-deficient systems that demonstrated the effects of in vivo metabolic modulation on Tfh cell responses, support the crucial roles of glucose metabolism in Tfh cell differentiation. Altogether, our study illustrates that mTORC1 and mTORC2 link immune signals to metabolic and transcriptional activities for Tfh cell differentiation.

Spontaneous Tfh cell generation maintains GC responses in PPs that contribute to mucosal IgA production (Fagarasan et al., 2010), but the underlying molecular pathways remain elusive. Our study links mTOR and cellular metabolism to Tfh cell differentiation in PPs. Whereas both mTORC1 and mTORC2 are important in this process, mTORC2 is selectively required for proper T cell localization in PPs, expression of Foxo1 targets, and nuclear exclusion of Foxo1 upon ICOS ligation. Reduced Tfh cell differentiation in Rictor-deficient mice could be partially restored by deletion of one allele of Foxo1, highlighting an ICOS-mTORC2-Foxo1 signaling axis in Tfh cell responses. Of note, whereas previous work in CD8+ T cells associates mTORC1 with T cell migration (Finlay and Cantrell, 2011; Finlay et al., 2012), our results here reveal a function of mTORC2 in mediating CD4+ T cell accumulation specifically in PPs. Also, in contrast to other effector T cell subsets in which mTORC1 generally plays a dominant role relative to mTORC2 (Chi, 2012), mTORC2 plays a more prominent role in Tfh cell differentiation and immune homeostasis in PPs. These findings highlight the unique signaling requirements of Tfh cells.

A recent report has found that shRNA-mediated silencing of mTOR or Raptor enhances Tfh cell frequency following...
adoptive transfer of retrovirus-transduced cells and LCMV infection (Ray et al., 2015). The exact reasons behind the divergent observations from ours are unclear, but efficiency of gene silencing might be critical and gene dosing effects might lead to different results. Additionally, silencing of mTOR exerts a strong inhibitory effect on Th1 cell differentiation in LCMV infection.

Figure 6. Raptor and Rictor Are Required for ICOS-Mediated Signaling Events and Anabolic Metabolism

(A–D) Mice were immunized by NP-OVA plus LPS in alum, and splenic Tfh cells were analyzed for the expression of Bcl6 and IRF4 (A), ICOS (B, left), KIf2, IIr7 and Cc7 (C), and CD62L, CD127, and CD69 (D). (B, right) ICOS expression on Tfh cells from PPs under steady state.

(E) Naive CD4+ T cells from WT, OX40creRptorfl/fl or OX40creRictorfl/fl mice were activated by anti-CD3 and anti-CD28 for 3 days, rested for 3 hr, and re-stimulated with anti-ICOS. Phosphorylation of S6 and Akt at Ser 473 was examined by flow cytometry at indicated time points.

(F–H) Metabolic assays in activated T cells that were re-stimulated with indicated stimuli for 24 hr, with the final 4 hr labeled with 14C-acetate to measure de novo lipogenesis (F and G) or with [3-^3H]-glucose to measure glycolysis (H).

(I) Glucose uptake as measured by 2-NBDG labeling in Tfh cells in spleen from WT, OX40creRptorfl/fl, and OX40creRictorfl/fl mice at 7 days after NP-OVA immunization.

(J) Frequencies of GC and Tfh cells in spleen from WT and OX40creMycIfl/fl mice at 7 days after NP-OVA immunization.

(K) Frequencies of GC and Tfh cells in PPs from WT and OX40creMycIfl/fl mice under steady state. *p < 0.05, **p < 0.01, ***p < 0.001 (Mann-Whitey test). Data are representative of two (A, C–J) or at least three (B and K) independent experiments. Error bars represent SEM. See also Figure S6.
Figure 7. Glucose Metabolism Promotes Tfh Cell Differentiation

(A and B) Purified CD4+ T cells were polarized in vitro to generate activated T cells (Tact), Tfh-like, Th1, and Th17 cells for high-resolution metabolomics analyses. The top 250 differentially observed metabolites are shown by heatmap (A) and principle component analysis (B).

(C) T cells were polarized in vitro to generate Tfh-like cells, in the presence of vehicle, 2-DG (250 μM), or rotenone (5 nM), for 3.5 days, followed by analysis of IL-21 expression. The fold change in the number of IL-21+ CD4+ cells in comparison to vehicle treated cells is shown.

(D and E) T cells were activated or polarized in vitro to generate Tfh-like cells for 3.5 days, followed by analysis of Glut1 expression by flow cytometry (D; right, the MFI of Glut1) or immunoblot (E).

(F) Flow cytometry of Glut1 expression on Tfh (B220−CD4+PD−1+CXCR5+) and non-Tfh cells (B220−CD4+PD−1−CXCR5−) from PPs. Right shows the MFI of Glut1.

(G) T cells from WT or Slc2a1-Tg mice were polarized in vitro to generate Tfh-like cells. After 3 days, the cells were treated with vehicle (DMSO) or rapamycin (50 nM) for 20 hr, followed by analysis of IL-21 expression. Graph represents the fold change in the number of IL-21+ CD4+ cells as a result of rapamycin treatment compared with that of vehicle treatment.
suggested that Thf and Th1 cells could have different sensitivity to reduced mTOR activity. We also note that our results using PTEN-deficient or PI3K over-activation mice are consistent with previous studies that show a positive role of PI3K signaling in Thf cell differentiation (Gigoux et al., 2009; Rolf et al., 2010).

Recent studies highlight distinct metabolic requirements for different T cell states and fates (Maclver et al., 2013). Our studies show that Thf cells utilize and require oxidative metabolism, glucose uptake and glycolysis are also important and can promote Thf cell differentiation in vivo. Bcl6, the master regulator for Thf cells, has been shown to inhibit glycolysis in vitro (Oestreich et al., 2014). Compared to Th1 cells, Thf cells exhibit lower rates of glycolysis and oxidative phosphorylation (Ray et al., 2015). Conversely, Bcl6 expression and Thf cells are associated with elevated cell proliferation (Kitano et al., 2011; Lüthje et al., 2012), demonstrating a use of anabolic metabolism. We show here that Thf cell differentiation required the interplay between immune signaling from ICOS-mTOR or Thf cell-promoting cytokines and metabolic reprogramming. This is reminiscent of the role of IL-2-mTORC1-mediated metabolism in orchestrating Treg cell proliferation and suppressive activity (Zeng et al., 2013). Our conclusion is further supported by results from inhibition of Myc or increased Glut1 expression, which directly illustrate the crucial role of anabolic metabolism in Thf cell differentiation. Thf cells might utilize this mixed metabolism to support both proliferation and cellular longevity while not depleting nutrients in GCs.

In summary, our study has identified the crucial roles of mTORC1 and mTORC2 in linking ICOS signals to metabolic and transcriptional regulation as a key mechanism for Thf cell differentiation under various contexts. Thf cells play an important role in humoral immunity but also contribute to autoimmune diseases. Our results suggest that clinical manipulation of Thf cells could be accomplished by targeting mTOR signaling or metabolic pathways for therapeutic intervention of immune-mediated diseases.

**EXPERIMENTAL PROCEDURES**

**Animals**

All mice were kept in specific pathogen-free condition in Animal Resource Center at St. Jude Children’s Research Hospital and Duke University. All animal protocols were approved by appropriate Institutional Animal Care and Use Committees. Further information is included in the Supplemental Experimental Procedures.

**Tfh-like Cell Culture**

Tfh-like cells were generated as described (Awe et al., 2019). Naive CD4+ T cells were isolated by magnetic beads and cultured on anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml)-coated plates in RPMI-1640 media supplemented with 10% FBS, sodium pyruvate, penicillin/streptomycin, HEPES, and β-mercaptoethanol for 3.5 days. The following cytokines were added to generate Tfh-like subset: 100 ng/ml IL-6 (eBioscience), 50 ng/ml IL-21 (R&D Systems), 10 μg/ml of each anti-IL-2, anti-IFN-γ (eBioscience), anti-IL-4 (eBioscience, clone 11B11), and anti-TGF-β (eBioscience) (Awe et al., 2015). In some experiments, cells were treated with 5 nM rotenone, 250 μM 2-DG, rapamycin (50 nM), or DMSO vehicle.

**Flow Cytometry**

For analysis of surface markers, cells were stained in PBS containing 2% fetal bovine serum (FBS). Flow cytometry data were acquired on LSRII or LSR Fortessa (BD Biosciences) or MACSQuant (Miltenyi) and analyzed using Flowjo software (Tree Star). Further information is included in the Supplemental Experimental Procedures.

**Immunofluorescence**

Fresh frozen PPs were cryosectioned, stained with antibodies, and imaged using Nikon TIE inverted microscope and an EMCCD camera (Andor). To analyze Foxo1 intracellular localization, we fixed, permeabilized, and stained cells with antibodies, imaged using a Marians spinning disk confocal microscope (Intelligent Imaging Innovations) and EMCCD camera, and analyzed using Slidebook software (Intelligent Imaging Innovations). Details are included in the Supplemental Experimental Procedures.

**Fecal IgA Measurement**

Fecal pellets were collected, weighed, and resuspended in PBS with complete protease inhibitor (Roche) at 100 μg/ml. The tubes were taped horizontally on a vortex and shake vigorously for 10 min. The suspensions were centrifuged at 400 g for 5 min to pellet large debris. Supernatants were filtered through a 70 μm cell strainer, and the flow-through was collected and centrifuged at 8,000 g for 5 min. Supernatants were collected and IgA concentration was measured by ELISA (eBioscience).

**ICOS Stimulation**

After 3 days activation, live T cells were purified using Ficoll. Cells were rested for 3 hr and then incubated with anti-ICOS (5 μg/ml; C398.4A; Biolegend) followed by crosslinking with goat anti-hamster antibody (Jackson ImmunoResearch).

**Metabolic Flux Analysis**

Glycolytic flux was determined by measuring the detritiation of [3-3H] glucose (Shi et al., 2011). De novo lipogenesis was measured as described before (Zeng et al., 2013). Details are included in the Supplemental Experimental Procedures.

**Metabolomics**

Metabolomic analyses were performed using LC Q Exactive Mass Spectrometer (LC-QE-MS) (Thermo Scientific) (Liu et al., 2014). MetaboAnalyst was used to analyze range-scale data and provide PCA and KEGG pathway analysis of metabolites significantly changed (1.5-fold difference, p < 0.05) (www.metaboanalyst.ca/).

**Gene-Expression Profiling**

Details are included in the Supplemental Experimental Procedures.

**Statistical Analysis**

p values were calculated with unpaired Student’s t test, Mann-Whitney test, or analysis of variance (GraphPad Prism) as specified in figure legends, with proper post-test analysis performed.

**ACCESSION NUMBERS**

The microarray data are available in the Gene Expression Omnibus (GEO) database under the accession number GSE85555.
SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.08.017.

AUTHOR CONTRIBUTIONS

H.Z. designed and performed experiments with Raptor, Rictor, PTEN, and Myc mutant mice and wrote the manuscript; S.C. designed and performed Glut1 mutant mouse and metabolic experiments and wrote the manuscript; C.G. performed imaging assays; G.N. performed bioinformatics analyses; S.S. performed LCMV infection; S.A.B. and C.C. performed immunoglobulin ELISA; R.J.K. assisted with immune experiments; X.G. and J.W.L. performed metabolomics analyses; B.Y., M.D., and M.O.L. provided reagents and insights; and J.C.R. and H.C. designed experiments, wrote the manuscript, and provided overall direction.

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