Mammalian Target of Rapamycin Protein Complex 2 Regulates Differentiation of Th1 and Th2 Cell Subsets via Distinct Signaling Pathways

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DOI 10.1016/j.immuni.2010.06.002

SUMMARY

Many functions of the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) have been defined, but relatively little is known about the biology of an alternative mTOR complex, mTORC2. We showed that conditional deletion of rictor, an essential subunit of mTORC2, impaired differentiation into T helper 1 (Th1) and Th2 cells without diversion into FoxP3+ status or substantial effect on Th17 cell differentiation. mTORC2 promoted phosphorylation of protein kinase B (PKB, or Akt) and PKC, Akt activity, and nuclear NF-κB transcription factors in response to T cell activation. Complementation with active Akt restored only T-bet transcription factor expression and Th1 cell differentiation, whereas activated PKC-θ reverted only GATA3 transcription factor and the Th2 cell defect of mTORC2 mutant cells. Collectively, the data uncover vital mTOR-PKC and mTOR-Akt connections in T cell differentiation and reveal distinct pathways by which mTORC2 regulates development of Th1 and Th2 cell subsets.

INTRODUCTION

So that specific needs for T cell help in immunity can be met, naive CD4+ T cells can differentiate into functionally distinct subsets of effector and regulatory (Treg) T cells after activation (Glimcher and Murphy, 2000; Zhu and Paul, 2008). This flexibility allows modulation of antigen-specific responses and adaptive immunity against microbes. Among these subsets, T helper 1 (Th1) cells produce cytokines such as IFN-γ after activation and IL-12 and IFN-γ after exposure to signals elicited by intracellular microbes (Glimcher and Murphy, 2000). A Th2 cell subset is induced by different cues and produces a distinct program of cytokines (IL-4, -5, and -13) for allergic and antiparasitic responses. Several more effector states can develop from naive CD4+ T cells: IL-17-producing Th17, induced Treg, IL-9-producing Th9, and IL-21-producing follicular helper (Tfh) cells (Locksley, 2009). Although the balance among these CD4 subsets is crucial, much remains unknown as to how signals are integrated to determine T cell fate and function.

T cell activation by antigen is essential for the development of effectors from naive T cells, and this process is strongly potentiated by engagement of costimulatory receptors on the T cells. CD28 dramatically enhances Th1 or Th2 cell responses (Kane et al., 2001; Kuchroo et al., 1995). Similarly, inducible costimulators such as ICOS and OX40 strongly enhance Th2 cell development and Th1 cell responses (Lane, 2000). Thus, costimulation of T cell receptor (TCR) signaling is vital for efficient development of several CD4+ T cell effector states. Furthermore, the precise quantitative and qualitative signaling elicited by the TCR or costimulators can guide the balance of differentiation into the different T helper subsets (Constant and Bottomly, 1997). As such, signaling molecules activated by TCR and costimulation are likely to be of vital importance in identifying means of manipulating the properties of immune responses.

Key molecules activated by costimulation include the mammalian Target of Rapamycin (mTOR), protein kinase B (PKB, also known as Akt), and protein kinase C (PKC)-θ (Huang et al., 2002; Lin et al., 2000). Upon TCR engagement and CD28 ligation, PKC-θ is phosphorylated and enhances T helper responses, in part by promoting nuclear translocation of NF-κB transcription factors (Coudronnire et al., 2000; Wang et al., 2004). In parallel, TCR engagement with costimulation also increases phosphatidylinositol 3-kinase (PI3K) activity. PI3K increases amounts of phosphatidylinositol (3,4,5)-triphosphate (PIP3), which recruits the PI3K-dependent kinase (PKD) 1 and activates Akt via phosphorylation of a conserved residue termed Akt (T308) (Scheid et al., 2002). Among its molecular targets, Akt leads to activation of mTOR (Kane and Weiss, 2003).

The importance of understanding of how specific signaling pathways impact T cell physiology is underscored by the successes and toxicities of immune suppressant drugs such as rapamycin, which targets mTOR. Rapamycin can inhibit proliferation of conventional T lymphocytes without blocking Treg cells (Battaglia et al., 2005; Valmori et al., 2006) and appears to bias the acquisition of CD4+ T cell functions inasmuch as it represses Th1, Th2, and Th17 cell development while...
enhancing induced Treg cells (Blazar et al., 1998; Kopf et al., 2007). There are at least two independent pools of mTOR in mammalian cells, of which the first is an acutely rapamycin-sensitive assembly termed mTOR complex 1 (mTORC1) (Laplante and Sabatini, 2009). A major role for mTORC1 in T lineage cells is clear from the effects of rapamycin and loss-of-function models for Akt and PDK1 (Hinton et al., 2004; Juntilla et al., 2007). Thus, although mTORC1 can also be activated by Akt- and PISK-independent mechanisms (Carrière et al., 2008; Fang et al., 2001), an important linear pathway to mTORC1 via Akt is solidly established and the data establish Akt and mTORC1 as key transducers of proliferative and differentiative signals in the T cell lineage.

After its activation by PDK1, Akt can be further phosphorylated at a C-terminal hydrophobic motif (HM) termed S473 (Scheid et al., 2002). This modification executed by PDK2 activities increased the cell-free in vitro activity of T308-phosphorylated Akt (Chan and Tsichlis, 2001; Scheid et al., 2002). PKC-β, DNA-PK, and integrin-linked kinase can provide PDK2 function in particular cell types (Dong and Liu, 2005). More recently, a second multiprotein complex that includes mTOR, mTORC2, has been identified as a major PDK2 (Sarbassov et al., 2005). In contrast to mTORC1, relatively little is known about the biological roles and functions of mTORC2 (Laplante and Sabatini, 2009). mTORC2 requires unique components—rictor and SIN1—that are not shared by mTORC1 (Jacinto et al., 2006). Loss of mTORC2 is embryonic lethal, but specific cellular or physiological targets in development are unknown (Guertin et al., 2006; Shiota et al., 2006). Intriguingly, mTORC2 was vital for Akt phosphorylation at T308 as well as S473 in multiple cell lines (Sarbassov et al., 2005) but not in rictor- or SIN1 null mouse embryonic fibroblasts (MEFs) (Guertin et al., 2006; Jacinto et al., 2006). Overall, it remains unclear whether mTORC2 impacts Akt activity or its targets in T cells. Apart from Akt, mTORC2 can phosphorylate subunit rictor. The principal role revealed for rictor after CD4+ T cell activation was promotion of differentiation into Th1 and Th2 cell subsets. In contrast, mTORC2 inhibited the induction of regulatory T cells under permissive conditions, but the defective cells exhibited no diversion from T helper cell differentiation into FoxP3+ status or marked abnormality of Th17 cell generation. Biochemical analyses revealed that mTORC2 deficiency impacted both Akt activity and phosphorylation of conserved PKC motifs. Strikingly, constitutively active Akt rescued T-bet expression and Th1 but not Th2 cell differentiation, whereas PKC-βII led to a reciprocal outcome. Thus, each of these mTORC2 targets selectively suppressed distinct defects of rictor-deficient cells, revealing an important branchpoint downstream from this signal-transducing complex in T cells.

RESULTS

Costimulation and T Cell Activation Induce Rictor-Dependent Akt HM Phosphorylation

We first analyzed the relationship of TCR costimulation to Akt phosphorylation and mTORC2 activity by using combinations of anti-CD3 and anti-CD28. Anti-CD28, alone and synergistically with anti-CD3, increased phosphorylation not only at Akt T308 but also at the Akt S473 (Figure 1), indicating that costimulation induced PDK2 activity. Costimulation also increased the phosphorylation of p70 ribosomal S6 protein kinase (S6K1) at T389, a site modified by mTORC1. mTORC2 also mediates serum-induced PKC phosphorylation at turn (TM) and hydrophobic (HM) motifs (Guertin et al., 2006; Ikenoue et al., 2008), and we found CD28 stimulation enhanced PKC-β-mediated PKC HM and TM phosphorylation (Figure 1).

To identify roles of mTORC2 in mature T cells, we used conditional inactivation of rictor. Deletion driven by Cre early in the T cell lineage (i.e., starting at the DN2 stage) decreased...
developmental efficiency and led to substantial phenotypic abnormalities of the resultant T cell repertoire (data not shown). Using a codon-optimized Cre cDNA that is directed to start expression after thymocyte progression to the double positive (DP) stage by the distal Lck promoter (‘‘dLck-iCre’’) (Zhang et al., 2005), we detected deletion in T cells, whereas floxed alleles were almost all intact in the thymus and in Thy1− cells from peripheral lymphoid organs (Figure S1A available online). Thymocytes from dLck-iCre+ Rictor fl/fl (abbreviated as cKO) mice and wild-type controls showed no abnormalities except for very modest decreases in CD8 SP cells and TCRhi HSAlo thymocytes (Figures S1B–S1D). Spleen and lymph node CD4+ T cell numbers were normal, whereas CD8+ T cells decreased slightly (Figure 2A) and frequencies of CD44hi, CD62Lhi, and CD25hi cells were similar in cKO and WT mice (Figures S1F–S1I). Thus, mature CD4+ T cells developed normally when rictor deletion was deferred during thymic differentiation, whereas a modest quantitative defect impacted production of CD8+ T cells.

Rictor Promotes a Restricted Set of T Helper Cell Fate Choices

Because rictor was vital for full Akt activity in T cells, we explored its impact on activation, differentiation, and function of T cells. We observed a dramatic decrease in Th2 cell differentiation among naive CD4+ T cells from cKO mice (Figures 3A and 3B). Comparisons of cKO to WT littermates also revealed impaired Th17 cell differentiation (Figures 3A and 3B). In contrast, no marked defect in Th1 cell development was observed (Figures 3A and 3B), indicating that rictor-containing complexes selectively promoted Th1 and Th2 cell differentiation.

Constitutively active Akt can inhibit Treg cell development (Haxhinasto et al., 2008), whereas rapamycin enhances their development at the expense of Th17 cell differentiation (Blazar...
et al., 1998; Kopf et al., 2007). cKO mice did not perturb the steady-state frequency of Treg cells (Figure S2A). However, FoxP3+ progeny developed from activated cKO CD4+ T cells supplemented only with TGF-β at a greater efficiency than controls (Figure 3Ca and Figure S2B). In contrast, normal frequencies of the FoxP3+ population were observed despite the mTORC2 defect when activated CD4+ T cells received strong T helper-differentiating signals (Figure 3D). These results show that mTORC2 inhibits iTreg cell differentiation under conditions already favorable to this process but rictor deficiency neither prevented Th17 cell development nor diverted developing effectors into a FoxP3+ population, from one representative experiment.

To test whether rictor influences an immune response in vivo, we immunized WT and cKO mice with keyhole limpet haemocyanin (KLH). The Ag-specific IgG2a response of cKO mice was significantly reduced, whereas IgG3 and IgG1 were not (Figures 3E and 3F and Figure S2C). T helper cell-derived IFN-γ is crucial for isotype switching to IgG2a, suggesting mTORC2 might be important for type 1 helper function. Consistent with this, the frequency of IFN-γ-producing CD4+ T cells was lower in lymphoid samples from cKO mice in an infectious challenge model (Figure 3G and Figures S2D and S2E). Moreover, total IgE production in rictor cKO mice was reduced again and amounts of serum IgE anti-ovalbumin were also reduced in the cKO mice (Figures 3H and 3I; Figure S2H). These results suggested a requirement for mTORC2 in both type 1 and type 2 T cell help to Ab class switching, and the aggregate findings indicate that rictor functions in vivo to mediate a subset of differentiation events and regulates the properties of T-B help.

**Rictor Promotes Proliferation without Impacting Survival**

The best-known functions of Akt are its suppression of apoptosis and acceleration of cell cycling (Manning and Cantley,
TCR-induced proliferation was attenuated in rictor-deficient T cells, at least partly because of a decreased rate of G1-S transitions (Figures 4A and 4B). Rictor-deficient CD8+ T cells as well as CD4+ T cells were defective; moreover, IL-4-dependent proliferation of both CD4+ and CD8+ T cells was also attenuated (Figures S3A–S3D). Cytokine receptors that share a common gamma chain signal protection against death by neglect, radiation-induced DNA damage, or etoposide-induced DNA damage. However, there was no decrease in survival rates of rictor-deficient T cells or increase in their apoptosis in the absence or presence of cytokines (Figures 4C–4E; Figure S3E and S3F). Similarly, we observed no sensitization to apoptosis after restimulation of activated CD4+ T cells (Figure S3G). Thus, mTORC2 mediates T cell proliferative signaling predominantly via cell cycle regulation rather than suppression of apoptosis.

Akt and PKC Mediate mTORC2 Regulation of T Helper Cell Differentiation

We transduced constitutively activated kinases targeted by mTORC2 into rictor-deficient T cells to determine the functional impact of Akt and PKC on the defects of Th helper cell differentiation. Th1 cell differentiation of cKO CD4+ T cells was rescued by the constitutively active mutant Myr-Akt (Figures 5A and 5B; Figures S4A and S4B). An Akt mutant activated by phosphomimetic residues at T308 and S473 [Akt(DD)] also suppressed the differentiation defect. In contrast, Akt(DA), which is refractory to S473 phosphorylation, did not raise the frequency of cKO Th1 cells (Figures S4A and S4B), Akt(DD) also restored normal proliferation to cKO Th1 cells (Figure S4D). Strikingly, however, Akt did not rescue the Th2 cell differentiation defect imposed by loss of mTORC2 function (Figures 5C and 5D). Thus, mTORC2 modification of Akt promotes Th1 cell differentiation but is not sufficient to explain the defect of Th2 cell differentiation.

Although mTORC2 phosphorylates PKCs at sites not thought of as major regulators of catalytic activity, we hypothesized that a PKC pathway might provide a functional link between mTORC2 and Th2 cell differentiation. Indeed, transduction of a constitutively active PKC-δ mutant (Liu et al., 2001) reverted the Th2 cell defect of cKO CD4+ T cells, yet did not impact the frequency of IFN-γ-positive cells (Figure 5A; Figures S4A and S4C). Akt and PKC-δ might collaborate as relays downstream of mTORC2, but cotransduction of these kinases into cKO CD4+ T cells showed only a small additive effect for Th2 cell differentiation (Figure S4E). As a mechanism for decreases in Th1 and Th2 cell differentiation when mTORC2 is compromised, amounts of the lineage-determining transcription factors T-bet and GATA-3 were substantially reduced in cKO CD4+ T cells (Figures 5E and 5F). Moreover, Akt restored WT amounts of T-bet expression to cKO T cells developing in Th1 cell conditions and failed to reverse the deficit of GATA3 in Th2 cell differentiation (Figures 5G and 5H). Conversely, constitutively active PKC-δ restored GATA3 but not T-bet under Th2 and Th1 cell conditions, respectively (Figures 5G and 5H).

Because mTORC2 differentially influenced fate choices of mature T cells, we explored relative activities of PDK1, PDK2, and mTORC2 in developing effectors. In comparing unpolarizing, Th1, Th2, and Th17 cell conditions, there were modest differences in amounts of PKC-δ and its HM-phosphorylated form and in ratios of Akt P-S473 to P-T308 (Figure 4F). STAT transcription factors guide choices for CD4+ T cells during T helper development and control the “master regulators” of Th1, Th2, and Th17 cell fates. However, tyrosine phosphorylation of the relevant STAT transcription factors was normal in cKO cells (Figure 6A; Figure S5A). P-STAT induction in cells subjected to inhibition of mTORC1 and mTORC2, or PI3K, was also normal (Figures S5B–S5E). It was intriguing that rapamycin strongly inhibited T cell proliferation despite activated Akt or PKC-δ expression, but also impacted Th1 and Th2 cell frequencies among the hypo-proliferative cells (Figures S5F–S5I). This suggests mTORC1 may also relay signals promoting these...
states. The results collectively show that mTORC2 drives Th1 cell differentiation via Akt, whereas PKC-θ serves as the relay for the Th2 cell fate, with each of these kinases exerting key effects independent from the STAT pathway.

**Targets Downstream from mTORC2 in T Cells**

The findings raised questions about how rictor affects biochemical targets of mTORC2, Akt, and PKC-θ to regulate the key transcriptional determinants of Th1 or Th2 cell fates. The Akt target FoxO1 represses cell cycling, enhances apoptosis, and has been identified as a regulator of T-bet and galectin-3, proteins that promote Th1 cell responses (Jiang et al., 2009; Ouyang et al., 2009). Consistent with the reductions in Akt activity and T-bet expression (Figures 2C, 2D, 5E, and 5F), P-FoxO1 was decreased in cKO T cells though an analogous phosphorylation of GSK-3β was unaffected (Figures 6B and 6C). There also was no significant decrease in phospho-S6K1 (Figure 6C), a target of mTORC1 regulated indirectly by Akt. GSK-3β(p-S9) and mTORC1 are targeted by other kinases in addition to Akt so the results with GSK-3β and S6K1 may be due to functional redundancy. However, the findings show that mTORC2 relays input signals that are nonredundant for FoxO1 as an Akt target. Because TCR-induced P-FoxO1 as well as T-bet were decreased in cKO T cells, we also measured expression of galectin-3 and found it was reduced in cKO T cells (Figure 6D).

Unlike the complete deletion in T cells (Kerdiles et al., 2009; Ouyang et al., 2009), decreased P-FoxO1 would only diminish repression by FoxO1, so some FoxO1 target genes might not be impacted by mTORC2 dysfunction. Indeed, mRNA expression was normal for the FoxO1 target genes pointed out in Figure S4 and the Experimental Procedures. Recent work on CD8+ T cells reported that PI3K regulates CD62L transcription by a rapamycin-sensitive pathway involving regulation of Klf2 (Sinclair et al., 2008). Although there was no difference in CD62L expression at the surface of freshly isolated CD4+ T cells, we observed marked reductions in the frequency CD62Llo CD4+ (Figure 7A) and CD8+ (Figure S6B) cells after activation of cKO T cells. However, neither Klf2 nor Sell (encoding CD62L) mRNA expression was significantly altered in CD4+ cKO cells (Figure S6A). Thus, mTORC2 impacts CD62L downregulation after T cell activation by a nontranscriptional mechanism. Apart from this identification of a role distinct from the rapamycin-inhibited...
pathway, the data show that mTORC2 transduces signals controlling mRNA expression for known FoxO1-regulated genes involved in Th1 cell differentiation.

mTORC2 phosphorylates PKC-α and regulates 3T3 and HeLa cell cytoskeletons (Jacinto et al., 2004; Sarbassov et al., 2004), but no role in T cells is known. Phosphorylation of PKC HM and PKC-θ TM—direct targets of mTORC2—also decreased in cKO T cells (Figures 6B and 6C). PKC-θ is essential for coupling TCR and CD28 costimulation to nuclear amounts of NF-κB and Th2 cell differentiation (Cannons et al., 2004; Marsland et al., 2004). Importantly, TCR-stimulated NF-κB amounts in nuclei of rictor-deficient CD4+ T cells were substantially decreased (Figure 7B). Moreover, TCR induction of a Carma1-dependent NF-κB-driven promoter was decreased in CD4+ cKO cells (Figure 7C), whereas transfection of cKO CD4+ T cells with the PKC-θ construct restored promoter activity to the amount of WT cells transfected with empty vector (Figure 7D). These data indicate that PKC-dependent NF-κB activity downstream from the TCR depends on mTORC2. Of note, in addition to decreased NF-κB transcription factors in the nuclei of cKO CD4+ T cells, Bcl-3 was also diminished (Figure 7E). This atypical IκB-like protein converts NF-κB1-p50 homodimers to transcriptionally active complexes, so that GATA3 and Th2 cell differentiation are decreased in Bcl-3-deficient T cells as well as NF-κB1-p50 null cells (Corn et al., 2005). Consistent with this, decreased GATA-3 expression in mTORC2-deficient developing Th2 cells were restored by activated PKC-θ (Figures 5F and 5H). PKC-θ also promotes increased binding of the integrin LFA-1 to ICAM-1 (Letischka et al., 2008). Consistent with a functional impact of mTORC2 on the PKC pathway in T cells, the activation-induced increase in LFA-1 binding to ICAM-1 was impaired in cKO T cells (Figure 7F). Taken together with the data on Akt-targeted FoxO1 and reversion results, the findings place Akt and PKC functions downstream from mTORC2 in T cells. Further, the data provide evidence of a functional dichotomy in T helper cell differentiation, such that Akt mediates mTORC2 regulation of Th1 cell development and PKC links mTORC2 to differentiation into Th2 cells.

**DISCUSSION**

We have shown that mTORC2 selectively promotes subsets of T helper cell differentiation and restrains acquisition of FoxP3 expression, but a defect of mTORC2 does not divert differentiating populations into a Treg cell-like fate. Thus, Ag-specific antibody isotypes driven by type 1 and type 2 help decreased in cKO mice, and proliferation-independent analyses revealed blocks to Th1 and Th2 but not Th17 cell differentiation. Dissection of mTORC2 effects on differentiation, survival, and cell cycling provided evidence of an unexpected hierarchy in the roles of this complex. T cell survival was unaffected and proliferation decreased only modestly despite decreased Akt activity, yet the effects on differentiation were substantial but selective. TCR-CD28-induced increases in phosphorylation of both Akt S473, a direct target of mTORC2, and the T308 residue, which is the most critical determinant of Akt activation by PDK1, were attenuated in cKO T cells. In addition, the defect of mTORC2 also weakened functional signaling to PKC. This biochemical bifurcation
enhance the population of Treg cells in Th17 cultures (Blazar et al., 1998; Kopf et al., 2007; Valmori et al., 2006). Loss of function for mTOR in T cells has been reported to mimic the effects of a FoxP3-regulated gene, but not Gata3, and a normal efficiency of Th1 but not Th2 cell differentiation. In contrast, PKC-δ sufficed to restore expression of FoxP3, a FoxO1-regulated protein, but not Gata3, and a normal efficiency of Th1 but not Th2 cell differentiation. In contrast, PKC-δ sufficed to restore expression of FoxP3, a FoxO1-regulated gene, but not Gata3, and a normal efficiency of Th1 but not Th2 cell differentiation. In contrast, PKC-δ sufficed to restore expression of FoxP3, a FoxO1-regulated gene, but not Gata3, and a normal efficiency of Th1 but not Th2 cell differentiation. In contrast, PKC-δ sufficed to restore expression of FoxP3, a FoxO1-regulated gene, but not Gata3, and a normal efficiency of Th1 but not Th2 cell differentiation.

TOR was identified as the key target of a clinically important immune suppressant drug rapamycin. Attention focused on the capacity of this agent to inhibit proliferation and promote apoptosis, but rapamycin was later shown to block Th1 cell development, inhibit Th2 and Th17 cell differentiation, and enhance the population of Treg cells in Th17 cultures (Blazar et al., 1998; Kopf et al., 2007; Valmori et al., 2006). Loss of function for mTOR in T cells has been reported to mimic the effects of rapamycin on Th helper proliferation and differentiation and to divert CD4+ T cells under helper-differentiating conditions into FoxP3+ cells (Delgoffe et al., 2009). Signals that can reverse these abnormalities are not known. The lack of mTOR was reported to cause potentially meaningful decreases of IL-4-induced STAT6 and IL-6-induced STAT3. It is intriguing to compare these findings to those in the rictor-deficient state. Rictor cKO T cells exhibited defects primarily of Th1 and Th2 cell differentiation and were not diverted into FoxP3+ status under any condition promoting T helper effector differentiation, although the prevalence of iTreg cell increased in response to TGF-β under otherwise neutral conditions. Moreover, no defect of STAT phosphorylation was detectable in either rictor-deficient T cells or those inhibited for PI3K or mTOR. Collectively, these data provide direct evidence that a rictor-deficient state leads to attenuation of Th1 and Th2 cell differentiation without any diversion into iTreg cell or apparent abnormality of STAT protein induction by tyrosine phosphorylation. Instead, mTORC2 targets Akt and PKC, and the activated kinases suffice to restore normal diversion into iTreg cell or apparent abnormality of STAT protein induction by tyrosine phosphorylation. Instead, mTORC2 targets Akt and PKC, and the activated kinases suffice to restore normal divergence into iTreg cell or apparent abnormality of STAT protein induction by tyrosine phosphorylation. Instead, mTORC2 targets Akt and PKC, and the activated kinases suffice to restore normal divergence into iTreg cell or apparent abnormality of STAT protein induction by tyrosine phosphorylation. Instead, mTORC2 targets Akt and PKC, and the activated kinases suffice to restore normal divergence into iTreg cell or apparent abnormality of STAT protein induction by tyrosine phosphorylation.
only minimally impair Th2 cell differentiation (Mora et al., 2003; Wang et al., 1997). Moreover, almost all signaling proteins ultimately prove to serve in several heterologous complexes and have multiple signal inputs. Although an alternative signaling complex (McDonald et al., 2008) including rictor might contribute to results reported with mTOR cKO T cells when mTOR is depleted, the molecular epistasis results with active Akt and PKC-δ show that targets of mTORC2 suffice to revert the main defects in T helper differentiation if mTORC1 is active. Because Akt can liberate Rheb, a signal-transducing GTPase and activator of mTORC1, from repression by a Tsc1/Tsc2 complex (Inoki et al., 2002), the attenuated Akt activation observed in rictor cKO T cells might suggest a complex circuit involving mTORC1 in the data. Consistent with rictor or Sin1 null MEFs, however, we observed normal activity of mTORC1 at least at the target kinase S6K1. Moreover, T cell-specific Rheb depletion did not phenocopy loss of mTOR in terms of repressing FoxP3 (other components of the Rheb-deficient phenotype are unknown) (Delgoffe et al., 2009). Analysis of mTORC2 inactivation along with the Rhex data suggests intriguing possibilities. These include that the threshold for mTORC1 to repress FoxP3 is very low, so that PI3K and Rictor-independent mTORC1 activation contributes to differences (Gwinn et al., 2008; Ha et al., 2006). Alternatively, Rheb may function in a signaling pathway that impacts FoxP3 in an mTORC1-independent manner, or each complex (mTORC1, mTORC2) may independently mediate FoxP3 repression. Thus, although our data directly show that mTORC2 function is vital for Th1 and Th2 differentiation, mTORC1 is likely independently to contribute to these processes.

Notwithstanding these issues pertaining to mTORC1, our data underscore new features of mTOR in T cells. These include mTORC2 as the predominant PDK2 activity driving costimulation-enhanced HM phosphorylation and Akt activity and the impact of this pathway on T-bet expression and Th1 cell differentiation. Intriguingly, measurements comparing WT, heterozygous (Cre+/-, fl/+), and homozygous cKO T cells found that Th2 cell differentiation was strongly reduced at the intermediate expression of mTORC2, whereas Th1 cell differentiation of heterozygous cells was almost normal (data not shown). Thus, mTORC2 may function as a scalar regulator of the balance among T helper subsets so that when Akt activity is less affected, Th1 cell differentiation is preserved. The findings also provide evidence of an Akt-independent, PKC-dependent role for rictor in T helper differentiation.

The cytoplasmic tail of CD28 stimulates PI3K activity by collaboration of two separable transducer motifs and enhances recruitment of both PDK1 and PKC-δ into the immunological synapse (Andres et al., 2004). The requirements for rictor in T helper differentiation is similar to one aspect of signaling down-stream from CD28 in that Myr-Akt reversed only the impairment of Th1 cell, but not Th2 cell, differentiation observed when CD28 was absent (Kane et al., 2001). In terms of Th2 cell differentiation, both CD28 and PKC-δ are vital determinants of the efficiency of Th2 cell differentiation (Andres et al., 2004). The finding that rictor-deficient cells have reduced TCR-induced NF-κB activity, GATA3, and Th2 cell differentiation reversible by constitutively active PKC-δ suggests that mTORC2 is a critical link connecting costimulation to PKC-δ and PI3K. Further, our data suggest that this role connects to a well-established pathway in which PKC-δ activates NF-κB after TCR-CD28 stimulation by mobilizing a Carma1, Bcl-10, MALT-1 signaling complex.

Expression of c-Maf, a potent regulator of Il4 transcription in Th2 cell responses, was also rictor dependent. Vav1 couples the TCR pathway to Maf (encoding c-Maf) (Tanaka et al., 2005), but PI3K-PKB regulation is normal in Vav-deficient T cells (Wood et al., 2006). IL-6 collaborates with this TCR signal to induce c-Maf via STAT3 (Yang et al., 2005). However, rictor cKO cells had normal STAT3 induction and were reverted by PKC-δ. Thus, mTORC2 probably relays signals essential for c-Maf induction by a pathway that collaborates with but is parallel to these previously established mechanisms. More generally, the data indicate that loss of mTORC2 impairs a subset of biological targets of mTOR in T cells and acts via protein kinases B or C without an impact on cytokine-induced Stat protein tyrosine phosphorylation. This selectivity suggests the possibility of alternate therapeutic windows for agents that would impair mTORC2 function, for disease states in which complete mTOR inhibition might add toxicities but not benefits.

EXPERIMENTAL PROCEDURES

Mice, Antibodies and Reagents

Rictorfl/fl mice were bred to a B6 background. For T cell lineage-specific deletion, Rictorfl/+ mice were crossed with transgenic mice expressing codon-optimized Cre recombinase (iCre) under control of the distal promoter of Lck gene (Zhang et al., 2005). Mice were maintained in ventilated microisolator cages in specified pathogen-free conditions and used in accordance with IACUC regulations of Vanderbilt University. Sources of chemical and biological reagents (cytokines, antibodies) detailed in Supplemental Experimental Procedures.

Cell Culture and Retroviral Transduction

CD4+ cells were purified by positive selection with CD4-conjugated magnetic beads (Miltenyi Biotec). Lymphoid cells were cultured, proliferation assays were performed, and viromes were generated after transfection of 4NX ectopic packaging cells with constructs of Akt(DD) and Akt(DA) mutants (T389D, S473D, or S473D A), Myr-Akt, and PKC-δ in MSCV-IRES-GFP and -Thy1.1 (MiG RV, MiT RV, respectively) as described (Corn et al., 2003). Activated CD4 T cells were “spininfected” and then cultured in Th1 or Th2 cell conditions. For in vitro T helper cell differentiation, lymph node cells were activated with plate-bound αCD3 plus soluble αCD28 and cultured with IL-2. Subset-specific culture additions were: anti-IL-4 and IL-12 (Th1 cell culture), αIL-12, αIFN-γ, and MIF-4 (Th2 cell culture), or TGF-β, IL6, IL-23, αIFN-γ, αIFN-γ (Th17 cell conditions). After 5 days, cells were restimulated with plate-bound 0.5 μg/ml αCD3 and soluble 0.5 μg/ml αCD28, stained for CD4, intracellular IFN-γ, and IL-4 as described (Corn et al., 2003), or restimulated to yield culture supernatants and measure cytokine production by ELISA. Details are tabulated in Supplemental Experimental Procedures.

Transfection and Luciferase Assays

Activated CD4 T cells (4 × 10^6/sample) were rested in IL-2-free medium for 5 hr and cotransfected with 20 μg of RE/AP reporter DNA, 2 μg of TK-prl (Promega), and, where indicated, 10 μg of Mscv or Mscv-PK-C-δ-KA with the Mouse T cells Nuclear Factor kit (Amaza). Transfectants were returned to culture 4 hr and restimulated (1 μg/ml αCD3 and 1 μg/ml αCD28) for 6 hr. Luciferase assays were performed with the Dual-luciferase kit (Promega). NF-κB-driven firefly luciferase activity was determined with a luminometer and normalized to Renilla luciferase activity.

Immunoblotting and Akt In Vitro Kinase Assay

Proteins in cell extracts were prepared and analyzed as described (Mora et al., 2003) with appropriate IR dye-conjugated second Abs and laser excitation/quantitative fluorescence detection (Odyssey Infrared Imaging System [Li-Cor]). To analyze cytosolic and nuclear proteins, previously activated T cells were washed and fractionated into cytosolic and nuclear portions as
described (Sun et al., 2000). To measure Akt activity, αAkt immunoprecipitates of lysate proteins were used for in vitro kinase assays of GSK-3β (S21/S9) phosphorylation in accordance with kit instructions (Cell Signaling Technology). For each sample, Akt kinase activity determined by immunoblotting was normalized to the Akt in immune complexes.

Flow Cytometry, Cell Division, and TUNEL Assays
Cells were stained with Ab, or stained, fixed, permeabilized, and then further processed for detection of intracellular epitopes, all as described (Corn et al., 2003). For division measurement, cells were fluorescently-labeled with CFSE (1 μM, 5 min), activated as described for TdR incorporation, cultured 48 hr, stained with anti-CD4 and anti-CD8, and analyzed by FACS. S phase entry rates were analyzed 24 hr after activation by plate-bound αCD3 (1 μg/ml) and soluble αCD28 (1 μg/ml) labeling with BrdU (8 hr), CD4 and CD8 staining, processing for DNAse digestion, BrdU staining, and flow cytometry analysis as described (Corn et al., 2003). For apoptosis assay, cells cultured in the presence or absence of 10 ng/ml IL-4 for 20 hr, with or without irradiation (2 Gy) in a 137Cs irradiator, were stained for surface markers, subjected to TUNEL assays, and analyzed by flow cytometry in comparison to control reactions lacking TdR.

Immunizations, ELISA, and ELISPOT Assays
Mice were injected intraperitoneally (i.p.) with 20 μg KLH in incomplete Freund’s adjuvant (IFA) and boosted (20 μg KLH in IFA) 2 weeks later. Sera collected 5 days after the boost (19 days after primary immunization) were analyzed by isotype-specific capture ELISA. For IgG1 and IgG responses, mice were immunized twice with 20 μg low-endotoxin ovalbumin (Hyglos GmbH, Germany) in alum (Inject, Pierce) at a 1 week interval. Sera collected 19 days after the first immunization were analyzed for ovalbumin-specific IgG, IgG1, and IgG by capture ELISA. Alternatively, mice were immunized intravenously with Listeria monocytogenes (5 x 10^7 colony-forming units/mouse), and analyzed for IFN-γ expression by FACS and ELISPOT assays 6 days after infection. ELISPOT assays from two independent experiments with n = 8 (WT) and 6 (cko) mice were performed by stimulating portions of the same experimental samples with 10 nM and 100 nM of the class II MHC-restricted LLO(310–321) peptide.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.06.002.

ACKNOWLEDGMENTS
This work was supported by NIH RO1A068149 and Vanderbilt Institutional Bridge Funds; additional acknowledgements are included in the Supplemental Information.

REFERENCES


Immunity

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