Mammalian Target of Rapamycin Complex 2 Controls CD8 T Cell Memory Differentiation in a Foxo1-Dependent Manner

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In Brief
Zhang et al. demonstrate that mTORC2 deficiency favors CD8 T cell memory differentiation during the primary antigen-specific T cell response to *Listeria* infection. The effects result from higher Foxo1 transcriptional activity without dampening effector functions. They also show enhanced recall responses by mTORC2-deficient memory CD8 T cells.

Highlights
- mTORC2 deficiency promotes MPEC generation and reduces SLECs during *Listeria* infection
- Primed mTORC2-deficient T cells have effector functions and produce higher IL-2 levels
- mTORC2-deficient memory CD8 T cells mount more potent recall responses
- mTORC2 deficiency promotes CD8 memory generation via nuclear accumulation of Foxo1
Mammalian Target of Rapamycin Complex 2 Controls CD8 T Cell Memory Differentiation in a Foxo1-Dependent Manner

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SUMMARY

Upon infection, antigen-specific naive CD8 T cells are activated and differentiate into short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). The underlying signaling pathways remain largely unresolved. We show that Rictor, the core component of mammalian target of rapamycin complex 2 (mTORC2), regulates SLEC and MPEC commitment. Rictor deficiency favors memory formation and increases IL-2 secretion capacity without dampening effector functions. Moreover, mTORC2-deficient memory T cells mount more potent recall responses. Enhanced memory formation in the absence of mTORC2 was associated with Eomes and Tcf-1 upregulation, repression of T-bet, enhanced mitochondrial spare respiratory capacity, and fatty acid oxidation. This transcriptional and metabolic reprogramming is mainly driven by nuclear stabilization of Foxo1. Silencing of Foxo1 reversed the increased MPEC differentiation and IL-2 production and led to an impaired recall response of Rictor KO memory T cells. Therefore, mTORC2 is a critical regulator of CD8 T cell differentiation and may be an important target for immunotherapy interventions.

INTRODUCTION

CD8 T cells play a crucial role in immunity to infection and cancer (Chang et al., 2014; Dudda et al., 2013; Kaech and Cui, 2012). Following an acute bacterial or viral infection, naive CD8 T cells are instructed by antigens, co-stimulatory molecules, and cytokines to undergo clonal expansion and functional differentiation into effector or memory T cells. Killer cell lectin-like receptor G1 (KLRG1) and interleukin 7 receptor α (IL-7Rα, CD127) surface markers discriminate short-lived effector cells (SLECs) from memory precursor effector cells (MPECs) (Chang et al., 2014; Kaech and Cui, 2012; Kaech et al., 2003; Sallusto et al., 1999; Wherry et al., 2003). The decision process is affected by inflammation, which triggers distinct transcriptional programs in primed CD8 T cells (Joshi et al., 2007). Multiple transcription factors have been identified to control CD8 effector or memory differentiation (Böttcher and Knolle, 2015; Chang et al., 2014; Kaech and Cui, 2012). The T box transcription factors T-bet and Eomesodermin (Eomes) represent two key transcription factors in orchestrating the SLEC or MPEC fate decision (Chang et al., 2014; Joshi et al., 2007; Kaech and Cui, 2012). In addition, B lymphocyte-induced maturation protein 1 (Blimp-1) and B cell lymphoma 6 (Bcl-6) are reciprocal regulators of CD8 effector and memory differentiation. Blimp-1 enhances the formation of terminally differentiated effectors by directly binding to Bcl-6 to repress its expression (Ji et al., 2011; Kallies et al., 2009; Rutishauser et al., 2009). Moreover, Id2- or Id3-deficient mice fail to generate short-lived effector or long-lived memory cells, respectively (Ji et al., 2011; Yang et al., 2011). However, the T cell intrinsic signaling pathways controlling cell fate decision between SLECs and MPECs remain poorly understood.

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase that integrates multiple environmental cues to regulate cell growth, proliferation, and metabolism (Betz and Hall, 2013; Shimobayashi and Hall, 2014). In mammalian cells, mTOR can form functionally distinct mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) multiprotein complexes. Raptor and Rictor are the core adaptor components of mTORC1 and mTORC2, respectively (Betz and Hall, 2013), whose deletion leads to loss of mTORC1 or mTORC2 integrity and activity (Bentzinger et al., 2008). mTORC1 has been shown to regulate both innate and adaptive immunity (Chen et al., 2013; Chi, 2012; Delgoffe et al., 2009; Powell et al., 2012; Waickman and Powell, 2012; Zhu et al., 2014). In T cells, mTORC1 integrates the signals from the T cell receptor
(TCR), co-stimulation, cytokines, and nutrients to regulate cell proliferation and differentiation (Zhang et al., 2012). Indeed, mTORC1 is required for Th1, Th2, Th17, and invariant natural killer T (iNKT) cell differentiation (Delgoffe et al., 2009, 2011; Shin et al., 2014; Yang et al., 2013; Zhang et al., 2014). Interestingly, pharmacological inhibition of mTORC1 by rapamycin in acutely Lymphocytic choriomeningitis virus (LCMV)-infected mice leads to increased magnitude and qualities of memory CD8 T cell differentiation (Araki et al., 2009; Pearce et al., 2009). Consistently, deletion of TSC1 or TSC2, the upstream inhibitor of mTORC1, drives stronger effector responses but impairs memory formation (Krishna et al., 2014; Shrestha et al., 2014). Recently, the Rictor containing mTORC2 or its downstream target serum- and glucocorticoid-regulated kinase (SGK1) have been shown to promote Th2 differentiation (Delgoffe et al., 2011; Heikamp et al., 2014). However, the role mTORC2 may have in shaping CD8 T cell effector and memory generation remains unknown.

RESULTS
Rictor-Deficient CD8 T Cells Mount Effective Effector Responses
To investigate the role of mTORC2 in shaping CD8 effector and memory differentiation, we crossed Rictor knockout (KO) mice onto an OT-1 background and tested how Rictor deficiency would affect the CD8 response to Listeria monocytogenes ovalbumin (LM-OVA) infection. Using this system, Zehn et al. (2009) showed that the strength of TCR-ligand interaction critically affects CD8 T cell expansion, contraction, and migration. Therefore, we transferred equal numbers of WT (Rictor$^{flx/flx}$; CD4-Cre$^-$) or Rictor KO (Rictor$^{flx/flx}$; CD4-Cre$^+$) OT-1 cells separately into naive hosts that were then infected with LM recombinants encoding the natural ligand SIINFEKL (N4) or one altered peptide ligand, SIITFEKL (T4), with about 100-fold lower functional avidity for the OT-1 TCR (Zehn et al., 2009; Figure 1A). A similar engraftment efficiency of transferred WT and Rictor KO OT-1 cells was confirmed 1 day later, before LM-OVA infection (data not shown). On day 7 after infection, Rictor KO OT-1 cells showed a 2-fold reduced frequency and absolute number of CD8 T cells (Figure 1B). Consistently, deletion of TSC1 or TSC2, the upstream inhibitor of mTORC1, drives stronger effector responses but impairs memory formation (Krishna et al., 2014; Shrestha et al., 2014). Recently, the Rictor containing mTORC2 or its downstream target serum- and glucocorticoid-regulated kinase (SGK1) have been shown to promote Th2 differentiation (Delgoffe et al., 2011; Heikamp et al., 2014). However, the role mTORC2 may have in shaping CD8 T cell effector and memory generation remains unknown.

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Figure 1. Rictor-Deficient CD8 T Cells Mount Effective Effector Responses to Infection
(A) Schematic of LM-OVA infection. 10, 000 WT or Rictor KO naive OT-1 cells were transferred into naive CD45.1 recipients, followed by injection of 2,000 CFUs of LM-OVA N4 or T4 i.v. (B) Flow cytometry analysis of CD8 and CD45.2 cells in the spleen and pLNs. (C) The percentages and absolute numbers of transferred WT or Rictor KO OT-1 cells harvested in the spleen (top) and pLNs (bottom) were quantified after N4 infection. (D) In vitro killing assay with Cr51 release or sorted WT or Rictor KO effector OT-1 cells on day 7 after infection were incubated with SIINFEKL peptide-pulsed (left) or -non-pulsed (right) EL-4 target cells at different ratios. (E-G) Representative overlay histograms and the mean fluorescence intensity (MFI) of granzyme B (E), perforin (F), or CD107a (G) expression on transferred WT or Rictor KO OT-1 cells on day 7 after LM-OVA N4 infection. In (E) and (F), the shaded gray histograms represent background levels in gated CD4 T cells. For CD107a, the shaded gray histogram represents WT OT-1 cells without SIINFEKL peptide stimulation. Data are representative of three or four independent experiments and are presented as the mean ± SEM (4–6 mice/group). **p < 0.01; ***p < 0.001; ns, not significant. See also Figure S1.
effector cells compared with the wild-type (WT) in response to either N4 or T4 stimulation in the spleen, liver, lung, and bone marrow (Figures 1B, 1C, and S1A–S1C; data not shown). However, the levels of transferred CD8 T cells in the peripheral lymph node (pLN) were comparable, suggesting that T cell trafficking might also be regulated by mTORC2 (Figures 1B and 1C). To determine whether the reduced CD8 T cell response in the absence of Rictor was due to decreased proliferation or increased apoptosis, we first measured Ki67 expression on day 5 after infection. As expected, N4 infection triggered higher Ki67 expression than T4, confirming the higher expansion capacity upon strong ligand stimulation (Figure S1D). Consistent with the reduced CD8 T cell levels, Ki67 expression in Rictor KO OT-1 cells was reduced compared with WT OT-1 cells after N4 infection (Figure S1D). In contrast, Rictor deficiency did not detectably affect the apoptosis rate of effector CD8 T cells (Figure S1E). These data suggest that mTORC2 is required for CD8 sustained proliferation and accumulation.

Next we examined whether Rictor-deficient CD8 T cells were capable of killing antigenic target cells. We found that Rictor KO effector cells could kill target EL-4 cells pulsed with SIINFEKL peptide as efficiently as WT effectors (Figure 1D). Granzyme B and perforin are the key effector molecules responsible for direct cytotoxicity of CD8 T cells. In line with comparable killing ability in vitro, both WT and Rictor KO effector cells expressed similar high levels of granzyme B and perforin (Figures 1E and 1F). Furthermore, CD107a expression, a degranulation marker correlated with cytolytic capacity, was strongly upregulated at the surface membrane of Rictor KO effector cells upon in vitro peptide restimulation (Figure 1G). Together, our data suggest that acquisition of CD8 T cell effector function is independent of mTORC2.

Rictor Deficiency Leads to Enhanced MPEC Generation while Reducing SLECs

Upon infection, antigen-primed CD8 T cells undergo differentiation into SLECs and MPECs. SLECs, defined as KLRG1hi CD127lo, undergo massive apoptosis during the contraction phase after the infection clears, whereas KLRG1lo CD127hi MPECs show increased potential to differentiate further into long-lived memory lineage cells. To examine whether mTORC2 regulates SLEC and MPEC commitment, we determined the expression profiles of KLRG1, CD127, and CD27 on WT or Rictor KO effector OT-1 cells at the peak of the response to primary infection. We found a significant reduction of KLRG1hi CD127lo SLECs in the absence of Rictor (Figures 2A and 1C). Consistent with the reduced CD8 T cell levels, KLRG1 expression in Rictor KO OT-1 cells was reduced compared with WT OT-1 cells after N4 infection (Figure S1D). In contrast, Rictor deficiency did not detectably affect the apoptosis rate of effector CD8 T cells (Figure S1E). These data suggest that mTORC2 is required for CD8 sustained proliferation and accumulation.

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The frequency of Rictor KO OT-1 cells with the CD62L⁺ CD44⁺ central memory phenotype, whereas WT OT-1 cells rarely harbor this phenotype at this early time point after infection (Figures 2A–2C). Additionally, the percentages of CD127⁺ CXCR3⁺ cells were also enhanced in the absence of mTORC2 (Figure 2A).

We also found a much higher intensity of CD62L, CD127, and CD122 expression on a per-cell basis in the absence of mTORC2 (Figure 2A; data not shown), indicating the potential for Rictor KO cells to respond better to IL-7 or IL-15 stimulation. The increased expression of CD62L and CCR7 was also confirmed at the transcriptional level (Figure S2A). It has been shown that Bcl-2 expression is higher in MPECs that will differentiate further into a long-lived memory lineage. Indeed, we found that Bcl-2 expression was much higher in Rictor KO CD8 T cells than that of WT counterparts (Figures 2D and 2E).

To exclude any effects from the inflammatory microenvironments in different hosts, we adoptively co-transferred mixtures of WT CD45.1/2⁺ OT-1 cells with Rictor KO CD45.2⁺ OT-1 cells at a 1:1 ratio into the same host. Even in this setting, higher MPEC and reduced SLEC frequencies were observed in both the spleen and pLNs (Figures S2B and S2C; data not shown). The increased proportions of the Rictor-deficient KLRG1lo CD127hi or CD62L⁺ CD44⁺ populations were even more striking in the host pLN (data not shown). Taken together, these findings support a CD8 T cell-intrinsic role of mTORC2 in promoting MPEC while inhibiting SLEC differentiation.

**Rictor Deficiency Leads to Enhanced Cell-Autonomous IL-2 Production in CD8 T Cells**

At the peak of the primary T cell response to acute infection, only a small fraction of effector CD8 T cells is able to produce IL-2 (Joshi et al., 2007; Sarkar et al., 2008). On day 7 after LM-OVA N4 infection, about 15% of WT effector CD8 T cells secreted IL-2 (Figures 3A–3D). Most intriguingly, we found a remarkably increased frequency of IL-2-producing, Rictor-deficient CD8 T cells (Figures 3B–3D). In contrast, they produced similar amounts of interferon γ (IFNγ) and tumor necrosis factor α (TNF-α) in response to Ova-N4 peptide stimulation as WT CD8 T cells (Figures 3C and 3D). Moreover, we found that IL-2 production was also increased significantly in Rictor KO effector cells in response to LM-OVA T4 infection (Figure S3). Similarly, we reproduced the finding that Rictor KO effectors from LM-OVA N4 infection produced higher levels of IL-2 even with in vitro OVA-T4 peptide stimulation (Figure S3). Moreover, higher IL-2 production was also observed in Rictor KO effectors from LM-OVA T4 infection in the presence of antigen.

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of OVA-N4 peptide stimulation in vitro (Figure S3), suggesting that higher IL-2 production in Rictor-deficient CD8 T cells was programmed regardless of infection with high- or low-affinity ligand. Enhanced IL-2 production in the absence of functional mTORC2 was confirmed further in co-transfer experiments (Figures 3E–3H). Indeed, although, in the WT CD45.1/2+ 1:1 WT CD45.2+ OT-1 control co-transfer, similar IFNγ, TNF-α, and IL-2 production was apparent upon peptide restimulation in vitro (Figures 3E and 3G), a significant increase in IL-2 production in the absence of Rictor was found in the WT CD45.1/2+ 1:1 Rictor KO CD45.2+ OT-1 transfer settings, whereas IFNγ and TNF-α secretion remained the same (Figures 3F and 3H). These results indicate that Rictor deficiency works in a CD8 T cell-intrinsic manner to selectively increase IL-2 production in response to antigen challenge.

**Rictor Deficiency Leads to Enhanced Long-Lived CD8 Memory T Cell Differentiation**

It has been reported that superior IL-2-producing MPECs differentiate more efficiently into long-lived protective memory T cells capable of self-renewal and rapid recall responses (Joshi et al., 2007; Sarkar et al., 2008). Given the enhanced differentiation into high IL-2-producing MPECs, we investigated whether Rictor deficiency could give rise to increased long-lived memory T cell differentiation. We found that the higher levels of KLRG1lo CD127hi or CD62L+ CD44+ CD8 T cells were maintained stably throughout the observation time of 4 months post-infection (Figure 4A). This was also confirmed in the co-transfer setting (Figures S3B and S3C). Seven weeks after primary infection, we indeed observed higher frequencies and absolute numbers of transferred Rictor KO OT-1 cells than of WT OT-1 cells in both the spleen and pLN, with a more striking increase in pLN (Figures 4C, 4E, and 4G).
Meanwhile, the absolute numbers of memory CD8 T cells in non-lymphoid tissues like liver and lungs were also increased slightly but significantly (Figure 4D). At this time point, we also confirmed a higher expression level of CD27, CD62L, and CD127 in the absence of Rictor, whereas the CD44 expression remained similar (Figure 4E). This increased CD62L and CD127 expression was still observed 7 months after primary infection (data not shown). Together, these data support increased memory CD8 T cell generation in Rictor-deficient T cells.

**Rictor KO Memory T Cells Mediate More Potent Recall Responses**

A unique feature of memory T cells is their ability to mediate an accelerated recall response with higher magnitude upon antigen re-exposure. To determine the role of mTORC2 in this process, we transferred similar numbers of WT or Rictor KO memory CD8 T cells sorted from mice 7 weeks after the primary challenge into naive recipients. The secondary hosts were challenged 1 day later with LM-OVA. On day 4 after infection, we found significantly increased frequencies and absolute numbers of Rictor KO OT-1 cells in the spleen and liver (data not shown) compared with WT cell transfer (Figures 4F and 4G), directly implying that Rictor deficiency increases potently functional memory CD8 T cells. Interestingly, recalled Rictor KO OT-1 cells retained higher CD62L expression. In addition, they upregulated CXCR3 expression better, which is important for homing to inflammation sites (Figure S4A). Furthermore, we did not observe any difference in the killing capacity between WT and Rictor KO secondary effector cells (Figure 4H). We also assessed cytokine production by memory T cells after antigen re-exposure. Rictor KO secondary effectors indeed produced similar amounts of IFN-γ and TNF-α upon restimulation (Figures 4I, S4B, and S4C). Together, these results show that Rictor-deficient memory T cells mount more potent recall responses while preserving WT-like immediate effector function.

**Enhanced CD8 T Cell Memory Formation in the Absence of Rictor Is Associated with Transcriptional and Metabolic Reprogramming**

The T box transcription factors T-bet and Eomes control the cell fate decision between SLECs and MPECs (Intlekofer et al., 2005; Rao et al., 2012). A higher T-bet/Eomes ratio drives terminal effector differentiation, whereas a higher Eomes/T-bet ratio favors memory formation. We therefore examined the expression pattern of T-bet and Eomes in WT and Rictor KO OT-1 cells on day 7 after LM-OVA infection. Unexpectedly, given the similar level of effector cytokine production, we observed a significant decrease in T-bet expression and concomitantly increased Eomes expression in Rictor-deficient cells (Figures 5A–5D). Furthermore, this higher Eomes, lower T-bet expression pattern in Rictor KO cells was maintained stably even at the contraction (day 14 after infection; Figures S5A and S5B) or memory phases.

**Figure 5. Rictor Deficiency Alters the Balance of T-bet and Eomes Expression.**

(A) Representative overlay histograms showing T-bet and Eomes expression on day 7 after infection.

(B) Representative overlay histograms showing T-bet and Eomes expression in the mix on transfer day 7 after infection.

(C and D) As shown in (A), the MFIs of T-bet (C) and Eomes (D) in the spleen are summarized.

(E) Representative overlay histograms showing Blimp1 and Bcl-6 expression on day 7 after infection in the spleen.

(F) The MFI of Blimp1 and Bcl-6 is summarized.

(G) On day 7 after infection, transferred OT-1 effector cells were sorted for real-time PCR analysis of Id2 and Id3 expression.

Data are representative of three or four independent experiments and are presented as the mean ± SEM (4–6 mice/group). *p < 0.05, **p < 0.01. See also Figure S5.
Blimp1 drives terminal differentiation into effectors cells, whereas Bcl-6 promotes MPEC differentiation (Ji et al., 2011; Rutishauser et al., 2009). However, we did not observe any detectable change in Blimp1 and Bcl-6 expression with Rictor deficiency (Figures 5E and 5F). Id2 and Id3 have been shown to control CD8 effector and memory differentiation (Ji et al., 2011; Yang et al., 2011). Consistent with increased memory formation, we found a slight but significant increase in Id3 expression at the transcription level, whereas Id2 expression remained comparable (Figure 5G).

Tcf-1, the downstream target of the Wnt signaling pathway, is required for CD8 memory generation and recall response (Jeannet et al., 2010; Zhou et al., 2010). Consistent with increased memory formation, we found a slight but significant increase in Id3 expression at the transcription level, whereas Id2 expression remained comparable (Figure 5G).

In terms of metabolic activity, primed CD8 T cells switch from oxidative phosphorylation (OXPHOS) to glycolysis, whereas CD8 memory T cells, unlike effector or naive CD8 T cells, exhibit substantial spare respiratory capacity (SRC) in their mitochondria (van der Windt et al., 2012; Waickman and Powell, 2012). IL-15, a crucial cytokine supporting CD8 memory T cell generation, regulates SRC and oxidative metabolism by promoting carnitine palmitoyl transferase (CPT1a) expression (van der Windt et al., 2013). To understand the connection between CD8 effector or memory differentiation and metabolic changes brought about by dysregulated mTORC2 signaling, we primed WT or Rictor KO naive OT-1 cells with SIINFEKL peptide and IL-2 for 3 days and continued to culture in the presence of IL-2 or IL-15 for another 3-4 days to generate in vitro “effector” or “memory” cells, respectively. First, we confirmed the increased SRC with IL-15-treated WT CD8 T cells compared with IL-2 cultured cells (Figures S6A and S6B). Consistent with enhanced memory differentiation, Rictor-deficient OT-1 cells cultured in IL-15 showed increased SRC compared with the WT. Interestingly, even when cultured in the presence of IL-2, which promotes effector differentiation, Rictor KO cells showed a striking increase in SRC (Figures S6A and S6B). Memory CD8 T cells preferentially use mitochondrial fatty acid oxidation (FAO) for their energy supply (Pearce et al., 2009; van der Windt et al., 2013). By using etomoxir, an inhibitor of CPT1a, we observed a pronounced decrease in oxygen consumption rate (OCR) (an indicator of OXPHOS) in either WT or Rictor KO CD8 T cells cultured with IL-15, suggesting that these memory CD8 T cells highly depend on FAO (Figures S6C and S6D). In contrast, WT CD8 T cells cultured in IL-2 are less dependent on FAO to generate energy (Figures S6C and S6D).
Intriguingly, our results showed that, in contrast to WT CD8 T cells, Rictor KO CD8 T cells differentiated under effector conditions rely heavily on CPT1α-dependent fatty acid metabolism to sustain their OCR, a cardinal metabolic feature of memory T cells. A higher level of FAO was further confirmed by increased CPT1α expression in Rictor KO cells (Figure S6G).

To test whether mitochondria were involved in the metabolic reprogramming by mTORC2 deficiency, we first determined the mitochondrial mass by using MitoTracker staining for in vitro-cultured WT and Rictor KO OT-1 cells. Indeed, Rictor KO OT-1 cells showed increased mitochondrial mass compared with WT OT-1 cells (Figure S6E). In addition, the ratio of mitochondrial DNA versus nuclear DNA was also increased significantly in Rictor KO cells (Figure S6F). Pparγc-1α (PGC1α) plays a key role in mitochondrial biogenesis and function by inducing the expression of nuclear respiration factor 1 (Nrf1) and mitochondrial transcriptional factor A (Tfam). Consistent with the increased mitochondrial content, we observed an enhanced expression of PGC1α, PGC1β, and Tfam in Rictor KO cells (Figure S6G). Moreover, we found that the expression of several essential components of the mitochondrial respiratory chain complex was upregulated (data not shown). Together, these observations support the fact that Rictor deficiency might promote memory T cell metabolic features by increasing mitochondrial function.

**Increased Nuclear Foxo1 Accumulation Mediates Enhanced Memory Formation in Rictor-Deficient CD8 T Cells**

Foxo1 promotes CD8 memory generation by directly controlling Eomes and Tcf1 expression (Hess Michelini et al., 2013; Kim et al., 2013; Rao et al., 2012; Tejera et al., 2013). Moreover, it is known that Foxo1 is dispensable for a primary differentiation in a Foxo1-dependent manner. Knocking down Foxo1 with Foxo1 AAA-transduced GFP+ OT-1 cells showed reduced MPEC differentiation compared with the non-transduced GFP+ population (Figure 7B). In contrast, the scramble-transduced population did show MPEC differentiation similar to the untransduced internal controls (data not shown). Moreover, compared with scramble-transduced cells, Foxo1 shRNA-transduced OT-1 cells indeed produced less IL-2 (Figure 7C). Together, these results demonstrate that Foxo1 mediates increased MPEC differentiation and IL-2 production in Rictor-deficient CD8 T cells (Figure 7H). Consistently, Foxo1 knockdown (KD) reduced the expression of CD62L and CD127 (data not shown). This was also reflected by the upregulation of T-bet but decreased Eomes and Tcf-1 expression in Foxo1 KD cells (Figures 7D and 7E). More importantly, silencing of Foxo1 led to an impaired recall response of Rictor KO memory T cells. Untransduced Rictor memory KO CD8 T cells showed MPEC differentiation and IL-2 production in Rictor-deficient CD8 T cells (GFP+). Foxo1 AAA-transduced WT OT-1 cells showed higher FAO because administration of etomoxir largely reduced the oxygen consumption rate compared with untransduced cells (GFP). Interestingly, these Foxo1 AAA-transduced cells are even more dependent on FAO than the Rictor KO cells (Figures S7A and S7B).

**DISCUSSION**

In this study, we identify the mTORC2-Akt-Foxo1 signaling axis as a crucial regulator of CD8 T cell effector and memory differentiation via specific transcriptional reprogramming.

We show enhanced memory differentiation and better recall response upon re-challenge by CD8 T cells lacking Rictor. We further demonstrate that mTORC2 controls CD8 memory T cell differentiation in a Foxo1-dependent manner. Knocking down Foxo1 reverses the increased MPEC differentiation and IL-2 production in Rictor KO cells. Foxo1 regulates multiple facets of T cell biology by controlling proliferation, differentiation, and trafficking. In CD8 T cells, Foxo1 is dispensable for a primary response upon infection. However, memory formation and recall response were largely impaired in the absence of Foxo1. In the absence of mTORC2, increased Foxo1 retention in the nucleus of CD8 T cells drives enhanced Eomes and Tcf1 expression, which promotes CD8 memory differentiation (Figure 7H). Indeed, Foxo1 directly induced expression of both Eomes and Tcf1, as shown by chromatin immunoprecipitation sequencing experiments (Hess Michelini et al., 2013; Kim et al., 2013; Rao et al., 2012; Tejera et al., 2013). Moreover, it is known that Foxo1 acts as a repressor of T-bet expression in CD8 T cells (Rao et al., 2012; Tejera et al., 2013). Further studies are required to identify the downstream targets of Foxo1 in memory T cells.
Overexpression of Foxo1 inhibits T-bet expression, whereas Foxo1-deficient CD8 T cells show higher expression of T-bet (Rao et al., 2012). In line with these observations, we show much lower expression of T-bet in the absence of functional mTORC2. Silencing of Foxo1 restores T-bet expression while reducing Eomes and Tcf1 expression. Interestingly, upon rechallenge, secondary Rictor KO effector cells also show higher Eomes and lower T-bet expression (data not shown), indicating a higher possibility of enhanced secondary memory formation.

The involvement of mTORC1 in regulating CD8 memory differentiation has been suggested by administration of rapamycin and metformin (Araki et al., 2009; Pearce et al., 2009). In the case of rapamycin administration during acute LCMV infection, the enhanced memory differentiation could be phenocopied by silencing of Raptor in CD8 T cells (Araki et al., 2009). In marked contrast, Raptor KO CD8 T cells largely failed to generate effectors and also failed to generate a memory response (L.Z. unpublished data). This discrepancy suggests that fine-tuning of mTORC1, but not its ablation, is required for the enhancement of memory T cell formation. This remains to be addressed experimentally. Alternatively, however, out data raise the possibility that rapamycin may, in fact, exert its positive effect on memory T cell formation via the inhibition of mTORC2 signaling in CD8 T cells in addition to inhibiting mTORC1. Such cell type variation in the ability of rapamycin to inhibit mTORC2 has been shown recently (Wang et al., 2014).

Interestingly, Rictor KO CD8 T cells produce larger amounts of IL-2 compared with the WT during primary infection. At the memory phase, not only the frequency of IL-2-producing cells but also the intensity of IL-2 on a per-cell basis are largely increased (data not shown). These findings are in line with previous reports showing that autonomous IL-2 production by CD8 T cells during primary infection was critical for the subsequent mounting of an effective recall response (Feau et al., 2011; Williams et al., 2006).
In this regard, it remains to be addressed whether enhanced IL-2 production during a primary response in Rictor-deficient CD8 T cells accounts for the more potent recall response.

The effector function of Rictor-deficient CD8 T cells remained largely intact, as also shown in a recent study (Pollizzi et al., 2015). However, in contrast to that report showing an unchanged, if not enhanced, frequency of Rictor KO antigen-specific CD8 T cells, we observed a decreased accumulation of CD8 effector T cells with Rictor deficiency at the peak of the response to high- or low-affinity ligand-bearing Listeria. We discovered that this was mainly due to less sustained proliferation in the face of comparable apoptosis rates of WT or Rictor KO effectors. The discrepancy between the two studies may be due to different infection systems. Pollizzi et al. (2015) transferred 100-fold more OT-1 cells than we did and used Vaccinia instead of Listeria infection to convey the OVA antigen. Another difference is the use of slightly different Rictor-floxed mice in the two studies. Indeed, the Rictor-floxed mice from Pollizzi et al. (2015) were exon 3-flanked, whereas ours were exon 4- and 5-flanked (Bentzinger et al., 2008). Nevertheless, both Polizzi’s report and our report clearly demonstrate that effector functions such as cytokine production or killing capacity are unchanged upon Rictor deficiency, whereas enhanced memory responses can be induced.

It has become clear that CD8 T cell effector or memory differentiation processes are coupled with metabolic reprogramming. Unlike naive or effector T cells, memory CD8 T cells show higher SRC. In our study, we show that mTORC2-deficient CD8 T cells show increased SRC and FAO even in the presence of IL-2, indicating that metabolic reprogramming prevails even under conditions conducive to effector cell T cell differentiation, such as supplementation with IL-2 in activated T cell culture medium. We believe that the enhanced SRC and FAO in Rictor-deficient T cells were, at least in part, due to decreased Akt activity caused by mTORC2 functional deficiency. Indeed, we observed that a short period of Akt inhibition in vitro during CD8 T cell priming leads to enhanced generation of T cells with the memory phenotype (data not shown). More importantly, these Akt inhibitor pre-treated CD8 T cells show increased SRC and FAO, as determined by Seahorse analysis. Interestingly, we observed a significant increase in CPT1α expression in either Rictor KO CD8 T cells or WT CD8 T cells cultured in the presence of Akt inhibitor. Clearly, further exploration of mitochondrial function in mTORC2-deficient CD8 T cell differentiation is warranted.

SGK1 represents another key target downstream of mTORC2 signaling. Recently, SGK1 has been shown to promote Th2 differentiation (Heikamp et al., 2014). However, the role of SGK1 in CD8 T cells is unclear. Therefore, it remains important to address the possibility of SGK1 involvement in the regulation of transcriptional activity of Foxo1 and in CD8 memory formation. Foxo1 is a transcription factor that regulates genes controlling multiple cellular processes, including metabolism. However, Foxo1 activity seems to be tightly regulated by the exact amount of nuclear Foxo1, the availability of its co-activators/repressors, and post-translational modifications. It is conceivable that there might be differential requirements for Foxo1 during memory T cell differentiation and maintenance, important aspects that require further scrutiny.

Inhibition of mTORC1 or Akt correlates with increased memory formation and long-term persistence in vivo and enhanced tumor protection effects (Araki et al., 2009; Crompton et al., 2015; Kim et al., 2012; Pearce et al., 2009). We identified mTORC2 as a critical regulator of CD8 memory formation by orchestrating transcriptional and metabolic programming. mTORC2 signaling has typically been associated with control of cytoskeleton organization and cell adhesion and motility. These aspects need to be probed in responding Rictor-deficient CD8 T cells. In CD8 T cells, however, its role as a repressor of Foxo1 transcriptional activity suggests that the level of mTORC2 signaling in activated T cells has a direct effect on their ability to undertake the memory T cell differentiation pathway. Together, our findings raise the possibility that therapeutic inhibition of mTORC2 represents an effective strategy to generate robust memory responses against infections and cancer and but preserve potent effector functions.

EXPERIMENTAL PROCEDURES

Animals

Rictor<sup>fl/fl</sup> mice have been described previously (Bentzinger et al., 2008). T cell-specific Rictor KO mice were obtained by crossing Rictor<sup>fl/fl</sup> mice with transgenic mice that carried CD4 promoter-mediated Cre recombinase. All mouse strains were backcrossed to the C57BL/6J background. Rictor KO mice were further crossed on the OT-1 background. CD45.1 congenic C57/BL6 mice and CD45.1/2 WT OT-1 mice were maintained at the University of Lausanne’s specific pathogen-free facility. This study was approved by the Veterinary authority of the Canton Vaud (Permit 2688.0), and all experiments were performed in accordance with Swiss ethical guidelines.

OT-1 Cell Transfer and LM-OVA Infection

Naïve OT-1 cells were purified with a naïve CD8 isolation kit (STEMCELL Technologies). 10,000 CD45.2 naïve WT or Rictor KO OT-1 cells were then transferred into the naïve CD45.1 host intravenously (i.v.). For co-transfer settings, 10,000 CD45.1/2 WT naïve OT-1 cells were mixed at a 1:1 ratio with naïve OT-1 cells either from CD45.2 Rictor KO mice or CD45.2 WT littermates. Within 24 hr after OT-1 cell transfer, 2000 colony-forming units (CFUs) of LM-OVA N4 or T4 variants were administered i.v.

More experimental procedures can be found in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.095.

AUTHOR CONTRIBUTIONS


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