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Tuberous sclerosis complex 1 (Tsc1) enforces quiescence of naive T cells to promote immune homeostasis and function

Kai Yang[1], **Geoffrey Neale**[2], **Douglas R. Green**[1], **Weifeng He**[1], and **Hongbo Chi**[1]

[1] Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA

[2] Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA

Abstract

The mechanisms that regulate T cell quiescence are poorly understood. We report that tuberous sclerosis complex 1 (Tsc1) establishes a quiescence program in naive T cells by controlling cell size, cell cycle entry, and responses to T cell receptor stimulation. Loss of quiescence predisposed *Tsc1*-deficient T cells to apoptosis that resulted in loss of conventional T cells and invariant natural killer T cells. Loss of Tsc1 function dampened *in vivo* immune responses to bacterial infection. *Tsc1*-deficient T cells exhibited increased mTORC1 but diminished mTORC2 activities, with mTORC1 activation essential for the disruption of immune homeostasis. Therefore, Tsc1-dependent control of mTOR is crucial in establishing naive T cell quiescence to facilitate adaptive immune function.

Keywords

naive T cells; quiescence; apoptosis; immune response; mTOR

T lymphocytes are pivotal in adaptive immunity. Mature T cells circulate through the blood and peripheral lymphoid organs in a quiescent state (G0) characterized by decreased cell size and metabolic activity. Survival of naive T cells relies on the engagement of T cell receptors (TCRs) by self-peptide MHC complexes and the interaction between interleukin-7 (IL-7) and interleukin-7 receptor (IL-7R)[1–3]. As these signals can potentially result in inappropriate activation, T cell quiescence must be actively maintained[4–6]. Transcription factors Foxo and Klf2 were thought to regulate T cell quiescence by inducing the expression of inhibitors of cellular activation[7–9]. However, recent evidence suggests that they might not directly maintain the quiescent state; instead they contribute to T cell homeostasis by

Correspondence should be addressed to: Hongbo Chi, Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, 38105, USA. Phone: 901-595-6282; Fax: 901-595-5766; hongbo.chi@stjude.org.

AUTHOR CONTRIBUTIONS

K.Y. designed and performed cellular, molecular, and biochemical experiments and contributed to writing the manuscript; G.N. performed bioinformatic analyses; D.R.G. contributed genetic models and conceptual insights; W.H. contributed to cell purification; H.C designed experiments, wrote the manuscript, and provided overall direction.

regulating T cell survival and trafficking[10–15]. Therefore, it remains poorly defined how quiescence in T cells is established, and whether it is coordinately regulated with T cell survival.

When naive T cells encounter cognate antigen, they undergo extensive clonal expansion and switch their metabolic program from catabolism to anabolism. Despite adequate amounts of oxygen in the environment, activated T cells employ glycolysis to generate energy, a phenomenon known as the Warburg effect[16, 17]. The mammalian target of rapamycin (mTOR), a conserved Ser-Thr kinase, is a central regulator of glycolysis and cellular metabolism[18]. mTOR exists in two multi-protein complexes in metazoans, mTORC1 and mTORC2. mTORC1 contains the scaffolding protein Raptor and is partly sensitive to the immunosuppressant rapamycin. mTORC1 promotes translational initiation by phosphorylating S6 kinase (S6K) and 4E-BP1, and stimulates cell growth and metabolism. mTORC1 activity is stringently controlled by tuberous sclerosis complex 1 and 2 (Tsc1 and 2), the mutations of which are found in hamartoma syndrome that involves tissue overgrowth. Tsc1 and Tsc2 function as an integral complex, and ablation of either Tsc1 or Tsc2 disrupts the complex[18]. A second mTOR complex, mTORC2, contains a distinct scaffolding protein, Rictor, and is important for activation of Akt by inducing Ser473 phosphorylation and selective Akt targets such as Foxo[18]. There is a considerable interplay between the two mTOR complexes, and activated mTORC1 institutes negative feedback loops to inhibit Akt[18]. An essential role for mTOR in both the innate and adaptive immune systems is emerging[19–21]. In T cells, mTOR is an important determinant of lineage differentiation after antigen stimulation, but whether it is involved in the homeostasis of naive T cells is unclear.

Using a *Tsc1*-deficient murine model, we report here that Tsc1 is a central regulator of naive T cell quiescence and homeostasis. Loss of quiescence predisposed *Tsc1*-deficient T cells to apoptotic cell death and prevented effective generation of an adaptive immune response. These data point to a novel checkpoint active in naive cell quiescence that affects immune homeostasis and function. *Tsc1* deficiency resulted in increased mTORC1 but reduced mTORC2-Akt activities. Combined use of genetic models and pharmacological approaches indicated that Tsc1 regulates T cell homeostasis and function mainly by negative control of mTORC1 activity.

RESULTS

Role of Tsc1 in regulating peripheral T cell populations

mTOR is induced upon TCR engagement, but how it is regulated in naive T cells is unclear. Tsc1, a modulator of mTOR signaling, was expressed in thymic and peripheral naive T cells (Supplementary Fig. 1a–c). To investigate the function of Tsc1 in T cells, we crossed mice carrying a conditional *Tsc1* allele ($Tsc1^{flox/flox}$)[22] with *Cd4*-Cre transgenic mice to delete the floxed *Tsc1* allele specifically in T cells (hereafter referred to as "$Tsc1^{-/-}$ mice"). mRNA and protein analyses indicated efficient deletion of *Tsc1* in mature thymocytes and peripheral T cells (Supplementary Fig. 1a–c). Wild-type (WT) and $Tsc1^{-/-}$ mice contained similar numbers of total thymocytes as well as double-negative, double-positive, CD4 single-positive (CD4SP) and CD8 single-positive (CD8SP) subsets (Fig. 1a), and

comparable expression of thymocyte maturation markers including CD62L, CD69 and CD24 (Supplementary Fig. 1d). In contrast, *Tsc1*$^{-/-}$ mice contained drastically reduced T cells in the spleen (Fig. 1b), peripheral blood and lymph nodes and an altered CD4/CD8 ratio, compared to the WT or heterozygous (*Tsc1*$^{+/-}$) littermates (Supplementary Fig. 1e–g).

We next analyzed the distribution of various T cell subsets in the periphery of *Tsc1*$^{-/-}$ mice. Among the conventional CD4 and CD8 T cell compartments, *Tsc1*$^{-/-}$ mice contained an expanded CD62L^{lo}CD44^{hi} population with activated or memory phenotypes, with a corresponding reduction of CD62L^{hi}CD44^{lo} naive T cells (Fig. 1c). This alteration was further exacerbated in aged mice (Supplementary Fig. 2a). *Tsc1* deficiency did not appear to affect homeostasis of Foxp3-expressing T_{reg} cells (Supplementary Fig. 2b). In contrast, invariant natural killer T (iNKT) cells, as detected by the CD1d-PBS57 tetramer, were markedly reduced in the spleen and liver of *Tsc1*$^{-/-}$ mice (Fig. 1d and Supplementary Fig. 2c). Tsc1 is thus crucial for maintaining the peripheral populations of conventional T cells and iNKT cells.

To address whether the reduction of peripheral T cell pools in *Tsc1*$^{-/-}$ mice is a cell-autonomous defect, we generated mixed bone marrow (BM) chimeras by reconstituting *Rag1*$^{-/-}$ mice with a 1:1 mixture of WT or *Tsc1*$^{-/-}$ (CD45.2^{+}) BM cells and wild-type (CD45.1^{+}) BM cells. Compared with WT BM-derived donor T cells, *Tsc1*$^{-/-}$ donors in the reconstituted chimeras contained reduced CD4 and CD8 T cells and retained the increased CD4/CD8 ratio (Fig. 1e). iNKT cells were also diminished in the mixed chimeras (data not shown). Therefore, Tsc1 exerts a cell-autonomous function in maintaining peripheral T cell populations.

Tsc1 promotes survival of peripheral T cells

The overall size of the peripheral T cell pool is dependent upon T cell survival[1–3]. We therefore measured caspase activity, a hallmark of apoptotic cell death. *Tsc1*$^{-/-}$ T cells exhibited higher caspase activity, relative to WT controls, with a more prominent elevation in CD8 T cells (Fig. 2a). In contrast, mature thymocytes from WT and *Tsc1*$^{-/-}$ mice exhibited similar caspase activity (data not shown). Mitochondrial homeostasis is critical in regulating apoptosis, and loss of mitochondrial membrane potential is a general feature of apoptosis[23]. *Tsc1*$^{-/-}$ T cells exhibited decreased mitochondria content and loss of mitochondrial transmembrane potential, as revealed by staining with mitochondria-specific dyes[24] (Supplementary Fig. 3). Therefore, deficiency of *Tsc1* induces apoptosis of peripheral T cells.

We next evaluated the survival ability of *Tsc1*-deficient T cells *in vivo*. We adoptively transferred a mixture of equal numbers of WT or *Tsc1*$^{-/-}$ (CFSE-labeled donor) and CD45.1^{+} (spike) T cells into CD45.2^{+} recipient mice. The spike group served as an internal control for normalization, and sorted naive T cells (CD62L^{hi}CD44^{lo}CD25) were used to obviate the altered homeostasis developed in *Tsc1*$^{-/-}$ mice. In contrast to WT donor cells that were well maintained upon transfer, *Tsc1*-deficient cells were rapidly lost and essentially undetectable after 6 days (Fig. 2b). Similar changes were observed in the spleen, lymph nodes (Fig. 2b), and blood (data not shown), indicating that the disappearance of *Tsc1*$^{-/-}$ cells was unlikely to be caused by altered trafficking[25]. The presence of a normal

lymphocyte pool in the recipients prevented proliferation of donor cells in the time interval described. Therefore, disappearance of *Tsc1*-deficient T cells is most likely due to a defect in survival *in vivo*.

We then determined the intrinsic requirement for Tsc1 in the survival of naive T cells by examining IL-7 signaling[2, 3]. Sorted naive T cells from WT or *Tsc1*$^{-/-}$ mice were cultured with IL-7 for 3 days. Compared with WT cells, *Tsc1*$^{-/-}$ cells were much less responsive to IL-7 triggered survival effects (Fig. 2c). Expression of IL-7Rα was comparable between these two types of cells (Fig. 2c), suggesting that the defective response to IL-7 was not due to impaired IL-7Rα expression. These findings demonstrate that *Tsc1*$^{-/-}$ cells are defective in response to homeostatic cytokine signals *in vitro*.

To further understand the peripheral T cell deficiency observed in *Tsc1*$^{-/-}$ mice, we stimulated naive WT and *Tsc1*$^{-/-}$ T cells with anti-CD3, anti-CD3-CD28, or a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin. At 16 hours, while only a small subset of WT cells died, as indicated by their positive staining with propidium iodide (PI), the majority of *Tsc1*$^{-/-}$ T cells became PI-positive (Fig. 2d). Kinetic analysis showed that *Tsc1*$^{-/-}$ cells exhibited accelerated death after 6 hours of TCR stimulation, with CD8 T cells progressing slightly faster than CD4 T cells (Fig. 2e). T cells can die through both apoptotic and necrotic pathways [1]. Treatment with the anti-apoptotic compound Z-VAD attenuated cell death in *Tsc1*$^{-/-}$ cells (Fig. 2f). In contrast, treatment with necrostatin-1 (Nec-1) to block necrotic death had no noticeable effects (data not shown). Therefore, *Tsc1*$^{-/-}$ cells respond to TCR signals by undergoing extensive apoptotic death.

Tsc1$^{-/-}$ T cells die via the intrinsic apoptotic pathway

Apoptosis of T cells is mediated by the intrinsic apoptotic pathway (controlled by the balance of pro- and anti-apoptotic Bcl-2 family members) and the extrinsic pathway (controlled by signals delivered from death receptors such as Fas)[1–3]. Fas expression was comparable between WT and *Tsc1*$^{-/-}$ T cells (data not shown). We therefore focused on the intrinsic pathway, and found that Bcl-2 expression was downregulated in *Tsc1*$^{-/-}$ T cells (Fig. 3a). This was further examined by crossing *Tsc1*$^{-/-}$ mice with mice expressing a *Bcl2* transgene in lymphocytes (*Bcl2*-TG)[26]. Numbers of CD4 and CD8 T cells as well as iNKT cells were nearly completely restored in *Tsc1*$^{-/-}$ *Bcl2*-TG mice (Fig. 3b,c and Supplementary Fig. 4). Moreover, in response to TCR stimulation, the enhanced death of *Tsc1*$^{-/-}$ naive T cells was considerably reduced in *Tsc1*$^{-/-}$*Bcl2*-TG cells (Fig. 3d,e). These results demonstrate that the exacerbated death of *Tsc1*$^{-/-}$ T cells is mediated by the intrinsic apoptotic pathway.

The pro-survival function of Bcl-2 is dependent upon its ability to antagonize the pro-apoptotic Bcl-2 family members. Among them, the BH3-only protein Bim is important to initiate lymphocyte apoptosis[1]. *Tsc1*$^{-/-}$ naive T cells showed elevated Bim expression, and upon TCR stimulation, they retained high levels of Bim while WT cells downregulated Bim expression (Fig. 3f and Supplementary Fig. 5a). To determine whether Bim-dependent apoptosis accounted for the survival defect, we crossed *Tsc1*$^{-/-}$ mice to the Bim-deficient (*Bcl2l11*$^{-/-}$) background. The frequency of CD8 T cells in *Tsc1*$^{-/-}$ *Bcl2l11*$^{-/-}$ mice was elevated by more than two fold compared with *Tsc1*$^{-/-}$ mice (Supplementary Fig. 5b).

Although CD4 T cell percentage in *Tsc1*$^{-/-}$ mice was not strongly affected by Bim deficiency *in vivo*, TCR-mediated apoptosis of *Tsc1*$^{-/-}$ *Bcl2l11*$^{-/-}$ double knockout CD4 T cells was less pronounced compared with that of *Tsc1*$^{-/-}$ cells (Supplementary Fig. 5c). Therefore, Bim-dependent and -independent mechanisms trigger apoptosis when Tsc1 is absent.

We next assessed whether the survival defect of *Tsc1*$^{-/-}$ T cells was associated with increased production of reactive oxygen species (ROS), which have been implicated in T cell apoptosis[27]. *Tsc1*$^{-/-}$ T cells exhibited increased amounts of ROS (Fig. 3g). To test whether elevation of ROS in *Tsc1*$^{-/-}$ T cells contributes to their extensive apoptosis, we used N-acetylcysteine (NAC), a widely used antioxidant. Following treatment with NAC, the reduced frequencies of CD4 and CD8 T cells in *Tsc1*$^{-/-}$ mice were modestly but significantly restored (Supplementary Fig. 6a). Consistent with these findings, the excessive TCR-mediated apoptosis in *Tsc1*$^{-/-}$ T cells was partially suppressed in those from NAC-treated mice (Supplementary Fig. 6b). Collectively, both dysregulated Bcl-2 family proteins and elevated oxidative stress contribute to apoptotic cell death in *Tsc1*$^{-/-}$ cells.

Loss of quiescence and hyper-activation of *Tsc1*$^{-/-}$ cells

Homeostasis of naive T cells is believed to be contingent upon the maintenance of a quiescent phenotype[4–6]. Freshly isolated *Tsc1*$^{-/-}$ T cells consistently exhibited an increased cell size (Fig. 4a), suggesting a probable increase in cell growth. The cell growth event precedes DNA synthesis and is a prerequisite for cell cycle entry [28]. We therefore measured *in vivo* cycling of T cells using bromodeoxyuridine (BrdU) incorporation. Compared with WT cells, a greater percentage of *Tsc1*$^{-/-}$ T cells incorporated BrdU under steady-state conditions (Fig. 4b), indicative of an exit from quiescence.

Within the CD44^{hi} population, recently activated and memory-phenotype cells can be discriminated on the basis of expression of the IL-2 receptor β-chain (CD122) that is selectively expressed on memory-phenotype cells[6, 29]. In WT spleen, a large proportion of CD44^{hi} CD8 T cells expressed CD122, but in *Tsc1*$^{-/-}$ spleen, most CD44^{hi} CD8 T cells were negative for CD122 (Fig. 4c). Expansion of the CD44^{hi}CD122 population also occurred in CD4 T cells, although to a lesser extent (data not shown). In contrast, other T cell activation markers including CD69, CD25 and CD5 were not altered by *Tsc1* deficiency (data not shown). *Tsc1*$^{-/-}$ T cells thus exist in a semiactivated state *in vivo*.

Since *Tsc1*$^{-/-}$ mice possess a lymphopenic environment, it remains possible that the increased T cell activation was caused by homeostatic proliferation. To test this possibility, we used the reconstituted BM chimeras described above (Fig. 1e) to correct the lymphopenia. The elevation of the total CD44^{hi} population observed in *Tsc1*$^{-/-}$ mice was less pronounced in the mixed BM chimeras (Supplementary Fig. 7a), suggesting that lymphopenia-induced expansion contributes to such a phenotype. In contrast, the CD44^{hi}CD122^{-} population was selectively expanded in donor cells derived from *Tsc1*$^{-/-}$ BM cells, but not from WT or the co-transferred CD45.1^{+} cells (Fig. 4d). Moreover, *Tsc1*$^{-/-}$ cells in the chimeras retained the increases in cell size and cycling (Fig. 4e and Supplementary Fig. 7b). Therefore, the *in vivo* semiactivated state is not primarily driven by

the lymphopenic environment but is intrinsic to *Tsc1*$^{-/-}$ T cells, indicating a key role for Tsc1 to maintain T cell quiescence.

The loss of quiescence observed in *Tsc1*$^{-/-}$ naive cells *in vivo* suggests that they may have a greater response to acute *ex vivo* TCR signals. To test this hypothesis, we stimulated naive *Tsc1*$^{-/-}$ cells with TCR ligation and measured various parameters associated with T cell activation. *Tsc1*$^{-/-}$ cells showed much larger increases in cell size, ROS production and cycling than WT cells (Fig. 4f,g). Further, upregulation of the activation markers CD25, CD69 and CD71 was more prominent in *Tsc1*$^{-/-}$ cells (Fig. 4h). *Tsc1* deficiency thus promotes TCR-mediated activation of T cells.

Tsc1 establishes a quiescent gene expression program

We next used functional genomics to analyze the Tsc1-dependent gene signature in naive T cells. The expression profiles of *Tsc1*$^{-/-}$ CD4 and CD8 T cells showed similar changes, with both showing more upregulated than downregulated genes (Supplementary Fig. 8a). For these coregulated genes (Supplementary Table 1), gene ontology analysis revealed that one of the significantly regulated groups ($P = 8.04\times10^{-5}$) by Tsc1 in naive T cells is the cell cycle pathway. *Tsc1*$^{-/-}$ naive T cells had higher expression of cell cycle positive regulators including Cyclin A2, Cyclin B2, and E2F2 (Supplementary Fig. 8b), which would serve to synergistically promote cycling. To identify key networks regulated by Tsc1, we performed gene set enrichment analysis (GESA) on *Tsc1*$^{-/-}$ naive CD4 T cells [30]. *Tsc1*$^{-/-}$ T cells showed significant enrichment of 20 upregulated gene sets (with nominal *P* values equal to zero) that included a number of metabolic pathways. In addition, genes sets for cell cycle and apoptotic pathways were also upregulated in *Tsc1*$^{-/-}$ naive cells (Supplementary Fig. 8c). Therefore, *Tsc1* deficiency upregulates the metabolic and cell cycle pathways.

We next determined the dynamics of the response of *Tsc1*$^{-/-}$ naive T cells to TCR stimulation at the genome-wide level, by comparing gene expression profiles between WT and *Tsc1*$^{-/-}$ CD4 T cells stimulated with anti-CD3-CD28 for 4 hours (Supplementary Table 2). Gene ontology analysis showed that stimulated *Tsc1*$^{-/-}$ CD4 T cells upregulated a number of genes associated with T cell activation that encode cytokines and effector molecules (*Ifng*, *Il4*, and *Gzma*), cell surface molecules (*Cd24* and *Icam1*), signaling molecules (*Gadd45b* and *Gadd45g*), transcription factors such as *Eomes*, *Myb* (c-Myb), *Stra13* and cell cycle regulators. Conversely, genes known to be suppressed after TCR stimulation, especially *Il7ra* and *S1pr1*, were more profoundly downregulated in *Tsc1*$^{-/-}$ cells (Fig. 5a). Real-time PCR analyses confirmed altered expression of *Myb*, *Stra13*, *Ifng*, *Gzma*, *Gadd45g* and *S1pr1* (Fig. 5b). Thus Tsc1 establishes a quiescence gene expression program by linking cell cycle and metabolic machineries and expression of immune response genes in T cells.

Acute deletion of *Tsc1* abrogates T cell quiescence

To directly investigate the effects of Tsc1 on T cell quiescence, we crossed *Tsc1*$^{flox/flox}$ mice with *Rosa26*-Cre-ER^{T2} mice (a Cre-ER fusion gene was recombined into the ubiquitously expressed *Rosa26* locus) to generate *Tsc1*$^{flox/flox}$ *Rosa26*-Cre-ER^{T2} mice ("*Tsc1*-CreER mice" hereafter). Tamoxifen-mediated acute deletion of *Tsc1* allowed us to exclude the

contribution of potential secondary effects introduced by extensive T cell apoptosis, a lymphopenic environment, or continuous loss of Tsc1 throughout the lifespan of T cells. We treated WT and *Tsc1*-CreER mice with tamoxifen daily for a total of 4 days, and then rested them for 2 weeks. *Tsc1* was efficiently deleted in T cells (data not shown), associated with enlarged cell size (Fig. 6a) and more rapid cycling *in vivo* (Fig. 6b). After TCR stimulation, naive T cells from these animals upregulated cell size and CD25 more extensively than control cells (Supplementary Fig. 9a, b). Moreover, the short-term deficiency of *Tsc1* modestly affected peripheral CD8 T cell pools *in vivo* (Fig. 6c). In response to *ex vivo* TCR signals, *Tsc1*-deficient T cells were more susceptible to apoptosis than *Tsc1*-sufficient controls (Fig. 6d). A more sustained loss of Tsc1, induced by tamoxifen treatment followed by resting for 5 weeks, resulted in greater upregulation of cell size and reduction of peripheral CD4 and CD8 T cells, as well as the development of semi-activation phenotypes *in vivo* (Supplementary Fig. 9c–e). Therefore, inducible deletion of *Tsc1* in adult mice disrupts T cell quiescence.

Tsc1 enforces quiescence independently of cell survival

The results with inducible *Tsc1* deletion suggest that *Tsc1* deficiency abrogates T cell quiescence in the absence of overt apoptosis. To further explore the relationship between T cell quiescence and survival, we analyzed $Tsc1^{-/-}$ mice expressing the *Bcl2* transgene. Compared with *Bcl2*-TG mice, $Tsc1^{-/-}$ *Bcl2*-TG mice contained an expanded population of semiactivated ($CD44^{hi}CD122$) T cells (Fig. 6e). Also, T cells in these mice exhibited increases in cell size and cycling (Fig. 6f,g). After TCR stimulation, $Tsc1^{-/-}$ *Bcl2*-TG cells retained the phenotypes of increased cell size, hyper-production of ROS and greater upregulation of activation markers relative to the control *Bcl2*-TG cells (Fig. 6h). Therefore, defective survival and quiescence phenotypes in $Tsc1^{-/-}$ cells were uncoupled upon blockade of apoptosis. These findings, together with the results above (Fig. 6a–d), indicate that loss of quiescence is intrinsic to $Tsc1^{-/-}$ T cells and apoptosis is likely a consequence.

mTORC1 activation disrupts immune homeostasis

We determined the biochemical mechanisms by which Tsc1 controls T cell homeostasis. We first measured the effects of *Tsc1* deficiency on mTORC1 activity by examining S6K1, S6 and 4EBP1 phosphorylation[18]. Compared with WT naive CD4 T cells, $Tsc1^{-/-}$ cells showed increases in both basal and TCR-induced activities of these conventional mTORC1 targets (Fig. 7a), indicating an inhibitory role of Tsc1 on mTORC1 activity. We next measured mTORC2 activity by analyzing Ser473 phosphorylation of Akt[18]. $Tsc1^{-/-}$ T cells exhibited decreased phosphorylation of Akt (Ser473) and of Foxo1 and Foxo3, two mTORC2-Akt targets[18] (Fig. 7b). Similar alterations in mTORC1 and mTORC2 activities were observed in $Tsc1^{-/-}$ CD8 T cells (Supplementary Fig. 10a). Interestingly, markedly elevated mTORC1 activity was also observed in $Tsc1^{-/-}$ CD4SP thymocytes (Supplementary Fig. 10b), despite the lack of defects in their survival or homeostasis. Thus, the physiological function of Tsc1 may be context dependent, with the effects influenced by the developmental stages of T cells or environmental cues received by T cells.

We next dissected the relative contribution of the two mTOR complexes to immune homeostasis. We administered the mTORC1 inhibitor rapamycin to WT and $Tsc1^{-/-}$ mice

for 3–5 days. As expected, rapamycin treatment reduced mTORC1 activity (Supplementary Fig. 11a). Although such a short period of treatment was ineffective to affect peripheral T cell pools (Supplementary Fig. 11b), rapamycin blocked the cell size increase of *Tsc1*$^{-/-}$ T cells (Fig. 7c). When rapamycin-treated *Tsc1*$^{-/-}$ naive T cells were transferred into WT recipients, their survival was substantially improved (Fig. 7d). Further, upon TCR stimulation, these cells were considerably rescued from the enhanced death (Fig. 7e) and cell size upregulation (Fig. 7f). Therefore, the increased mTORC1 activity in *Tsc1*$^{-/-}$ cells is required to drive the excessive apoptosis and activation.

To determine the sufficiency and requirement of the diminished mTORC2-Akt activities in *Tsc1*$^{-/-}$ T cells for immune homeostasis, we used two approaches. First, we analyzed mice deficient in *Rictor*, the loss of which abrogates mTORC2 activity[18]. Deletion of *Rictor* using the *Cd4*-Cre system (*Rictor*$^{-/-}$) ablated Akt phosphorylation at Ser473 and diminished Foxo1 phosphorylation (Supplementary Fig. 12a). *Rictor*$^{-/-}$ mice contained largely normal peripheral T cell pools except for a slight reduction of CD8 T cells (data not shown), consistent with recent reports[31, 32]. Deficiency of *Rictor* did not alter T cell size or responses to TCR, except that *Rictor*$^{-/-}$ CD8 T cells exhibited slightly increased apoptosis *in vitro* (Supplementary Fig. 12b–d). Therefore, loss of mTORC2 activity is insufficient to disrupt immune homeostasis. Second, to test whether the diminished mTORC2-Akt activity is required for *Tsc1*$^{-/-}$ phenotypes, we crossed *Tsc1*$^{-/-}$ mice with those expressing the active *Akt* transgene in T cells (*Akt*-TG)[33]. As expected, phosphorylation of the Akt target gene, Foxo1, was largely restored in *Tsc1*$^{-/-}$ *Akt*-TG mice (Supplementary Fig. 13a). However, the *Akt* transgene failed to rectify the disrupted T cell homeostasis and survival observed in *Tsc1*$^{-/-}$ mice (Supplementary Fig. 13b–d). Therefore, mTORC2 is neither necessary nor sufficient to abrogate T cell quiescence or survival. While it remains possible that the impaired Akt activity might contribute to the increased sensitivity of *Tsc1*$^{-/-}$ cells to apoptosis by accelerating the execution of cell death, ultimately it is not the driving force.

Having established a role for Tsc1 in T cell quiescence via mTORC1 inhibition, we next explored how the activity of the Tsc1 pathway is regulated. We first assessed whether Tsc2 expression was affected by *Tsc1* deficiency, as these two molecules function as an integral complex[18]. Indeed, *Tsc1* deficiency in T cells resulted in the loss of Tsc2, thereby ablating the entire Tsc1-Tsc2 complex function (Supplementary Fig. 14a). This finding, although not unexpected, allowed us to dissect how upstream signals were transduced to the complex by examining signal-induced phosphorylation of Tsc2[18]. Notably, Tsc2 activity can be positively and negatively regulated by phosphorylation at Ser1387 and Thr1462, respectively[18, 34, 35]. Tsc2 phosphorylation at Thr1462 was rapidly induced after T cell activation (Supplementary Fig. 14b), thereby relieving the inhibitory effects of Tsc1-Tsc2 complex on mTORC1 activity. Conversely, Tsc2 phosphorylation at Ser1387 was maintained at a high level in naive T cells but diminished after sustained TCR stimulation (Supplementary Fig. 14b). These results support a model in which the Tsc1-Tsc2 complex is inactivated by TCR signals to facilitate mTORC1 activation, but the complex function is maintained in naive T cells to keep mTORC1 in check.

Tsc1 regulates antigen-specific immune response *in vivo*

To investigate the roles of Tsc1 in T cell-mediated immune response *in vivo*, we infected WT and *Tsc1*$^{-/-}$ mice with recombinant *Listeria monocytogenes* expressing ovalbumin (LM-OVA). As compared with WT controls, CD8 T cells in the infected *Tsc1*$^{-/-}$ mice contained significantly reduced frequency and numbers of tetramer-positive, antigen-reactive cells (Fig. 8a,b). *Tsc1*$^{-/-}$mice also contained fewer IFN-γ-producing CD8 T cells after *ex vivo* restimulation with the OVA peptide (Fig. 8c,d). These findings demonstrate that Tsc1 is required for antigen-specific immune response *in vivo*.

Given the excessive apoptosis of *Tsc1*$^{-/-}$ T cells, we determined whether the requirement for Tsc1 in the immune response was due to its effect on cell survival. To this end, we analyzed the antibacterial immune response in *Tsc1*$^{-/-}$ *Bcl2*-TG mice that contained largely normal T cell numbers (Fig. 3b,c). Expansion of OVA-reactive CD8 T cells was impaired in these mice (Fig. 8a–d and Supplementary Fig. 15a), despite that the OVA-reactive cells exhibited a similar extent of apoptosis as *Bcl2*-TG control cells (Supplementary Fig. 15b). Thus, the effect for Tsc1 to promote immune responses can be largely separated from its role in T cell survival. We then examined whether *Tsc1* deficiency was associated with an intrinsic defect in effector T cell differentiation. Under optimal differentiation conditions *in vitro*, naive CD8 T cells from *Tsc1*$^{-/-}$ *Bcl2*-TG cells were able to differentiate into IFN-γ^{+} cells (with slightly increased expression on a per cell basis as compared with *Bcl2*-TG controls) (Supplementary Fig. 15c). Deficiency of *Tsc1* therefore dampens *in vivo* immune responses through a mechanism that is largely independent of T cell survival or intrinsic differentiation potential.

DISCUSSION

A long-standing question in immunology is how quiescence of naive T cells is established[4, 5]. Here we describe that Tsc1-dependent control of mTOR activity is critical to enforce naive cell quiescence. Loss of this control mechanism in *Tsc1*$^{-/-}$ T cells results in increased cell size, rapid cycling and exuberant TCR activation that predispose these cells to apoptotic cell death, suggesting that Tsc1 represents a *bona fide* factor to establish T cell quiescence. Furthermore, Tsc1 is important for antigen-specific immune response, a function largely independent of Tsc1-mediated survival effects. At the molecular levels, Tsc1 acts as part of the Tsc1-Tsc2 complex whose activity is dynamically regulated by TCR signals. Tsc1 enforces T cell homeostasis by controlling mTORC1 activity to suppress the metabolic and cell cycle machineries and to establish a quiescent gene expression program. We propose that Tsc1-dependent pathway is a key checkpoint in naive T cells by integrating extracellular signals to control mTOR activity, thereby coordinating T cell quiescence, survival and immune function.

Deficiency of *Tsc1* in T cells led to exit from quiescence and excessive apoptosis. Both effects were observed *in vivo* under lymphopenic and lymphoreplete conditions, and were further recapitulated in highly purified naive T cells *in vitro*. Are these two effects independent of each other, or causally linked? *Tsc1*$^{-/-}$ T cells partly relied on Bim-dependent and oxidative stress pathways to trigger apoptosis, and they were protected from death by the *Bcl2* transgene. Yet, these T cells retained the phenotypes of disrupted

quiescence, as shown by the expansion of semiactivated T cells and increases in cell size, cycling and response to TCR signals. By contrast, short-term rapamycin treatment reduced T cell size and TCR activation and more importantly, restored the resistance of *Tsc1*$^{-/-}$ cells to apoptosis. Therefore, failure of *Tsc1*$^{-/-}$naive T cells to engage the survival machinery stemmed from loss of quiescence, a notion further supported by transcript profiling and analyses of T cells upon acute *Tsc1* deletion. We conclude that Tsc1 actively maintains a quiescent program in naive T cells that renders them resistant to apoptosis. Notably, Tsc1 regulates production of innate cytokines[36], and *Tsc1* deficiency in hematopoietic stem cells results in rapid cycling and expansion but impaired hematopoiesis and self-renewal[37, 38]. In the thymus, while Tsc1 regulates mTOR activity, no survival defects were noticed in *Tsc1*$^{-/-}$ thymocytes. Indeed, except for under non-physiological conditions such as nutrient starvation and genome damage[39, 40], mTOR activity is generally thought to promote cell growth and survival in diverse cellular contexts[18]. In T cells, mTOR inhibition by rapamycin *in vitro* diminishes cell size but not survival[41]. Therefore, Tsc1 plays a hitherto unappreciated role in naive T cells to link their quiescence and survival, while the activity of the Tsc1-Tsc2 complex is downregulated upon TCR ligation to facilitate mTORC1 activation.

An equally surprising finding is that *Tsc1*-deficint T cells failed to mount an efficient antigen-specific immune response *in vivo*, despite their enhanced response to acute TCR stimulation. Such an effect was observed even when the increased apoptosis of *Tsc1*$^{-/-}$ T cells was largely blocked, suggesting that maintenance of naive T cell quiescence is important for a productive immune response. The precise mechanism by which *Tsc1* deficiency dampens the immune response is uncertain, but may involve premature activation of the cell cycle and metabolic machineries and transcriptional responses in *Tsc1*$^{-/-}$ naive cells before they receive proper TCR signals. While blocking mTOR activity blunts effector T cell differentiation[20], our results indicate that hyperactive mTORC1 is also detrimental to antigen-specific responses. These findings highlight the presence of a defined threshold of mTOR activity in naive T cells to engage a productive immune reaction. Notably, mTOR inhibits development of memory CD8 T cells[42–44]. From a metabolic perspective, both naive and memory cells are quiescent[17]. It is tempting to speculate that maintenance of a quiescent state is essential for the strength of immune responses mediated by both naive and memory T cells, and further study is required to test this notion.

Recent work on mTOR signaling in the adaptive immune system has centered on its effects on T cell differentiation[20]. mTOR has been shown to promote the generation of effector cells while inhibiting T_{reg} differentiation[26, 31, 32, 45, 46]. None of the reported mutants in the mTOR pathway exhibit the phenotypes in T cell quiescence or survival. To our knowledge, the genetic model exhibiting the most extensive similarity as *Tsc1*$^{-/-}$ mice is *electra*, an immunodeficient mouse line carrying a mutation in the gene encoding Schlafen-2 (Slfn2), a protein with no clear biochemical functions[6]. We also noted that memory-phenotype ($CD44^{hi}CD122^{+}$) cells were greatly decreased in *Tsc1*$^{-/-}$ mice, a defect resistant to Bcl-2-mediated rescue effects. Therefore, differentiation of *Tsc1*$^{-/-}$ cells into memory cells is likely blocked, consistent with the effects of rapamycin to promote memory development[42–44]. In contrast, loss of iNKT cells in *Tsc1*$^{-/-}$ mice resulted from impaired

survival, because the *Bcl2* transgene rescued this defect. Tsc1 therefore controls the homeostasis of different T cell populations via distinct mechanisms.

Persistent mTOR activation results in metabolic stress and is associated with diverse pathologies such as cancer and metabolic diseases[18]. Conversely, inhibition of mTOR by rapamycin prolongs life span and increases quality of life by reducing the incidence of age-related pathologies in various species ranging from worms, fruit flies to mammals[47]. As a parallel, aberrant mTOR activity due to Tsc1 functional loss in T cells results in exit from quiescence and exacerbated activation and apoptosis, which can be ameliorated by rapamycin treatment. Therefore, the adaptive immune system has evolved to adopt an evolutionarily conserved pathway to regulate the life span and function of naive T cells.

ONLINE METHODS

Mice

Tsc1^{flox}, C57BL/6, CD45.1^{+}, and *Rag1*$^{-/-}$ mice were purchased from the Jackson Laboratory[22]. *Rictor*^{flox} mice were obtained from NIH Mutant Mouse Regional Resource Center[48]. *Cd4*-Cre, *Bcl2*-TG, *Akt*-TG and *Rosa26*-Cre-ER^{T2} mice have been described previously[26, 33, 46]. Mice at 6–10 weeks old were used unless otherwise noted. BM chimeras were generated by transferring 1×10^7 T cell-depleted BM cells into sublethally irradiated (5 Gy) *Rag1*$^{-/-}$ mice, as described previously[26, 46]. Rapamycin treatment was described previously[26]. For bacterial infection, mice were injected intravenously with the *Listeria monocytogenes* strain LM-OVA ($3–5\times10^4$ cfu/mouse) and analyzed 6–7 days later. For tamoxifen treatment, mice were injected intraperitoneally with tamoxifen (2 mg/mouse) in corn oil daily on 4 consecutive days and analyzed at the indicated times. For NAC treatment, mice were fed with NAC (1 mg/ml; Sigma) in the drinking water with the solution replaced every two days. All mice were kept in specific pathogen-free conditions in Animal Resource Center at St. Jude. Animal protocols were approved by Institutional Animal Care and Use Committee of St. Jude.

Flow cytometry

For analysis of surface markers, cells were stained with antibodies (all from eBioscience) in PBS containing 2% (wt/vol) BSA, unless noted otherwise. PBS57-loaded mCD1d tetramer was obtained form the NIH Tetramer Facility. Foxp3 staining was performed per manufacturer's instructions (eBioscience). BrdU and Annexin V staining was performed per manufacturer's instructions (BD Biosciences). Caspase activity was measured using FITC-VAD-FMK per manufacturer's instructions (Promega). For intracellular cytokine staining, T cells were stimulated for 5 h with PMA/ionomycin or $OVA_{257–264}$ peptide (SIINFEKL, for LM-OVA infected mice) in the presence of monensin before staining per manufacturer's instructions (BD Biosciences). To stain mitochondria, lymphocytes were incubated with MitoTracker Green (20 nM; Invitrogen) or TMRM (20 nM; ImmunoChemistry Technologies) at 37°C for 20 min after staining of surface markers. ROS was measured by incubation with CM-H2DCFDA (10 μM; Invitrogen) at 37°C for 30 min after staining of surface markers. Flow cytometry data were acquired on an upgraded 5-color FACScan or LSRII (Becton Dickinson), and analyzed using FlowJo software (Tree Star).

Cell purification and cultures

Lymphocytes were isolated from the lymphoid organs and naive T cells sorted on a MoFlow (Beckman-Coulter) or Reflection (i-Cyt). Sorted naive T cells ($CD4^+CD62L^{hi}CD44^{lo}CD25^-$) were used for *in vitro* cultures in Click's medium (plus β-mercaptoethanol) supplemented with 10% FBS and 1% penicillin-streptomycin as described previously [26, 46]. For drug inhibitor treatments, cells were incubated with vehicle or Z-VAD for 1 hour before stimulation.

Gene expression profiling by microarray analysis

RNA samples were analyzed using the Affymetrix HT_MG-430_PM GeneTitan peg array and expression signals were summarized using the RMA algorithm (Affymetrix Expression Console v1.1). Differentially expressed transcripts were identified by ANOVA (Partek Genomics Suite v6.5) and the Benjamini-Hochberg method was used to estimate the false discovery rate. Gene lists were analyzed for enrichment of Gene Ontology and canonical pathways using the DAVID bioinformatics databases [49]. Gene set enrichment analysis (GSEA) within canonical pathways was performed as described [30].

RNA and immunoblot analyses

Real time PCR analysis was performed as described previously, using primer and probe sets from Applied Biosystems [26, 46]. Immunoblot was performed and quantified as described previously [26], using the following antibodies: p-S6, p-Akt (S473), p-4EBP1, p-S6K1, p-Foxo1, p-Foxo3a, p-Tsc2 (S1387), Tsc1 (all from Cell Signaling Technology), p-Tsc2 (T1462) (Novus Biologicals), and β-actin (Sigma).

Statistical analysis

P values were calculated using Student's *t*-test and ANOVA. *P* values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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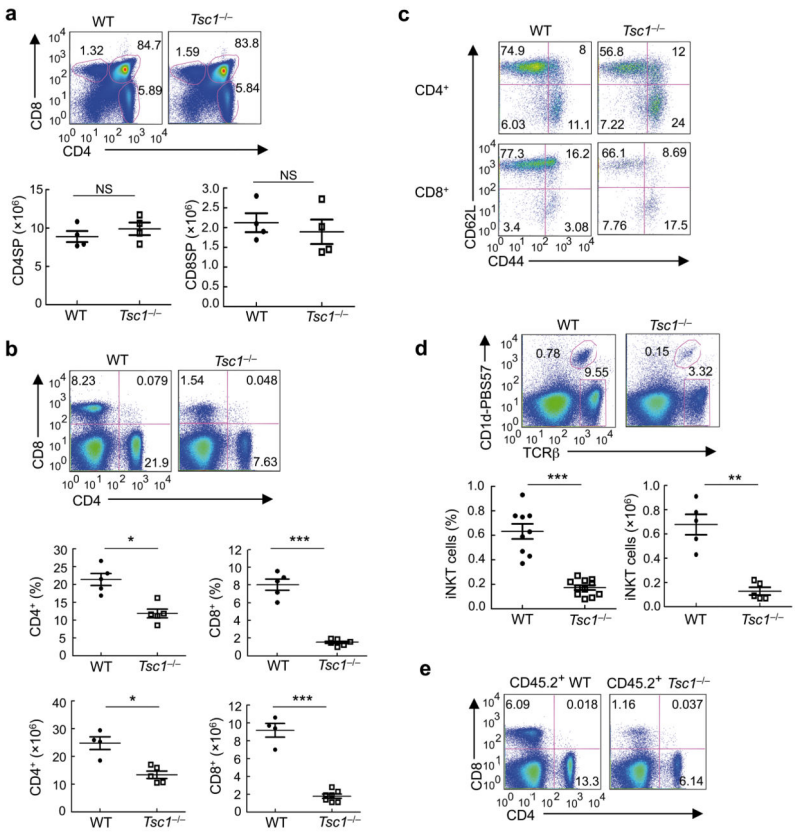

Figure 1. *Tsc1* deficiency leads to disrupted homeostasis of peripheral T cell pools

(**a**) Flow cytometry of thymocytes in wild-type (WT) and *Tsc1*$^{-/-}$ mice. Lower panels, numbers of CD4SP and CD8SP thymocytes (n=4–7). (**b**) Flow cytometry of CD4 and CD8 T cells in the spleens of WT and *Tsc1*$^{-/-}$ mice. Lower panels, proportions and absolute numbers of CD4 and CD8 T cells in the spleens of WT and *Tsc1*$^{-/-}$ mice (n=4–6). (**c**) CD62L and CD44 expression on splenic T cells of WT and *Tsc1*$^{-/-}$ mice. (**d**) Flow cytometry of iNKT cells (TCRβ^{+}CD1d-PBS57^{+}) in the spleens of WT and *Tsc1*$^{-/-}$ mice (n≥5). (**e**) Flow cytometry of CD4 and CD8 T cells in the spleens of mixed BM chimeras. BM stem cells from wild-type (CD45.1^{+}) and WT or *Tsc1*$^{-/-}$mice (CD45.2^{+}) were mixed at 1:1, and transferred into sublethally irradiated *Rag1*$^{-/-}$ mice to generate mixed BM chimeras, followed by analysis at 8 weeks after reconstitution. NS, not significant; * $P <$ 0.005; ** $P < 0.0005$; *** $P < 0.0001$. Data are representative of 4 (**a–d**) and 2 (**e**) independent experiments.

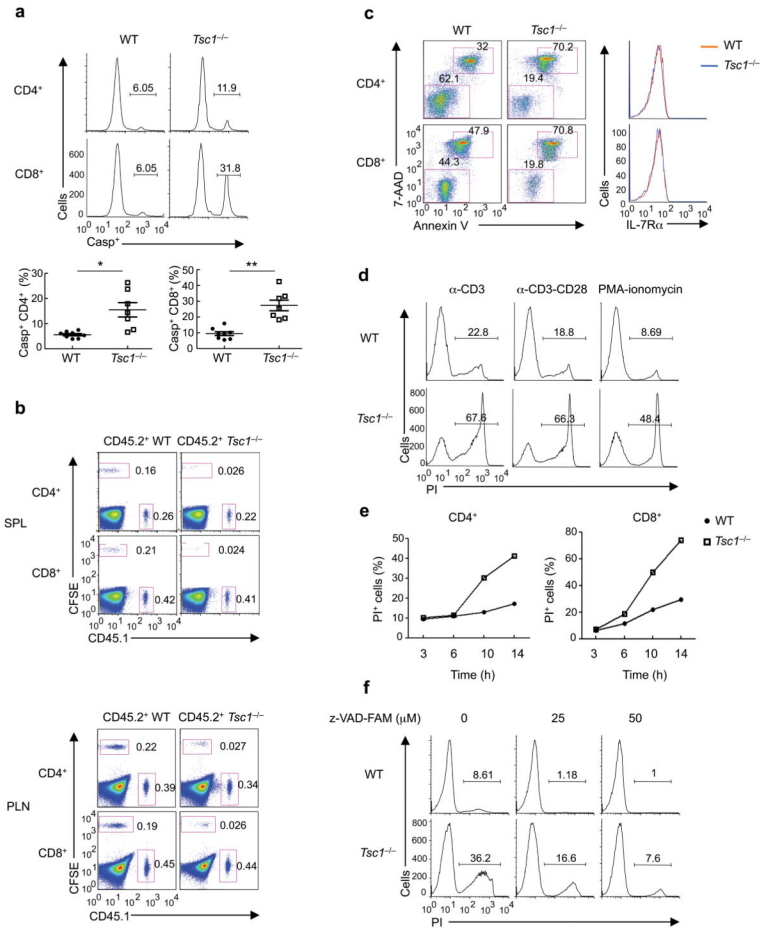

Figure 2. *Tsc1* deletion results in markedly elevated apoptosis of T cells

(**a**) Caspase activity by FITC-VAD-FMK staining in freshly isolated WT and *Tsc1*$^{-/-}$ T cells. Lower panels, proportions of caspase-positive (Casp^{+}) T cells (n=7–8). * P <0.005; ** P < 0.0005. (**b**) Flow cytometry of spleen (upper) and lymph node (lower) cells in the recipient mice (CD45.2^{+}) 6 days after adoptive transfer of equal numbers of CD45.1^{+} (spike) cells and WT or *Tsc1*$^{-/-}$ donor cells (CFSE-labeled). SPL, spleen; PLN, peripheral lymph nodes. (**c**) Annexin V and 7-AAD staining in naive WT or *Tsc1*$^{-/-}$ T cells cultured with IL-7 *in vitro* for 3days (left), and expression of IL-7Rα on freshly isolated cells (right). (**d**) Detection of PI^{+} apoptotic cells after 16 hours of stimulation of CD8 T cells with anti-CD3, anti-CD3-CD28, or PMA-ionomycin. (**e**) Kinetics of apoptosis (measured by frequency of PI^{+} apoptotic cells) after anti-CD3-CD28 stimulation of CD4 and CD8 T cells. (**f**) Detection of PI^{+} apoptotic cells in WT and *Tsc1*$^{-/-}$ CD8 T cells pretreated with vehicle or caspase inhibitor Z-VAD-FAM for 1 hour and then activated with anti-CD3-CD28 for 10 hours. Although not shown here, similar results were observed in CD4 T cells (**d,f**). Data are representative of 4 (**a,c**), 2 (**b,e**), 5 (**d**) and 3 (**f**) independent experiments.

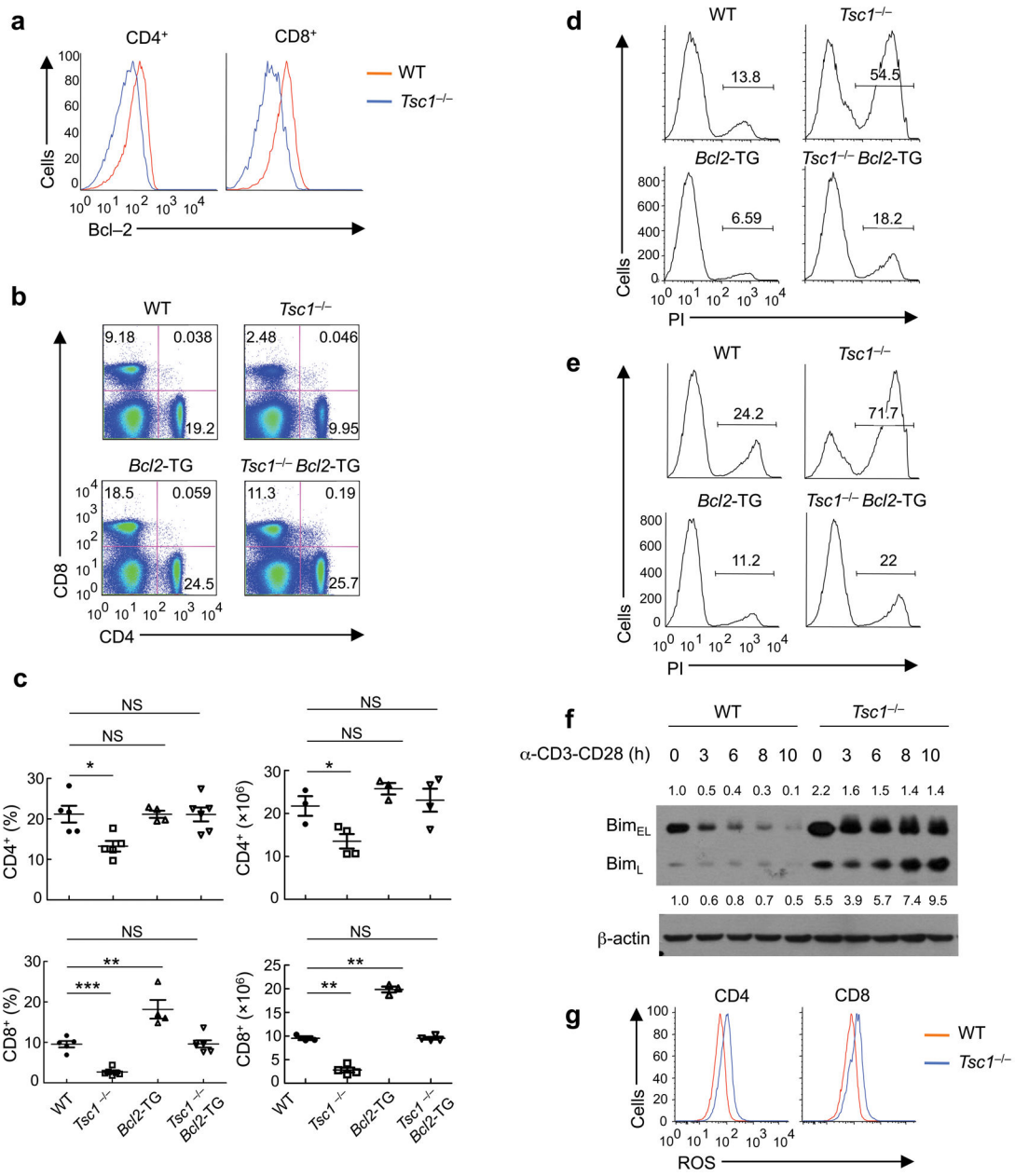

Figure 3. *Tsc1*-deficient T cells die via the Bcl-2 family-dependent intrinsic apoptotic pathway

(**a**) Intracellular Bcl-2 expression in WT and *Tsc1*$^{-/-}$ T cells. (**b**) Flow cytometry of CD4 and CD8 T cells in the spleens of WT, *Tsc1*$^{-/-}$, *Bcl2*-TG, and *Tsc1*$^{-/-}$ *Bcl2*-TG mice. (**c**) Proportions and absolute numbers of CD4 and CD8 T cells in WT, Tsc1$^{-/-}$, *Bcl2*-TG, and *Tsc1*$^{-/-}$*Bcl2*-TG spleens (n=3–6). NS, not significant; * P <0.05; ** P < 0.005; *** P< 0.0001. (**d,e**) Detection of PI^{+} apoptotic cells after 16 hours of stimulation of CD4 (**d**) and CD8 (**e**) cells with anti-CD3-CD28. (**f**) Expression of Bim_{EL} and Bim_{L} in naive and anti-CD3-CD28 activated WT and *Tsc1*$^{-/-}$ CD4 T cells. Numbers above (Bim_{EL}) and below (Bim_{L}) lanes indicate band intensity relative to that of the loading control-actin. (**g**) ROS

production in freshly isolated WT and *Tsc1*$^{-/-}$ T cells. Data are representative of 3 (**a,d–g**) and 4 (**b,c**) independent experiments.

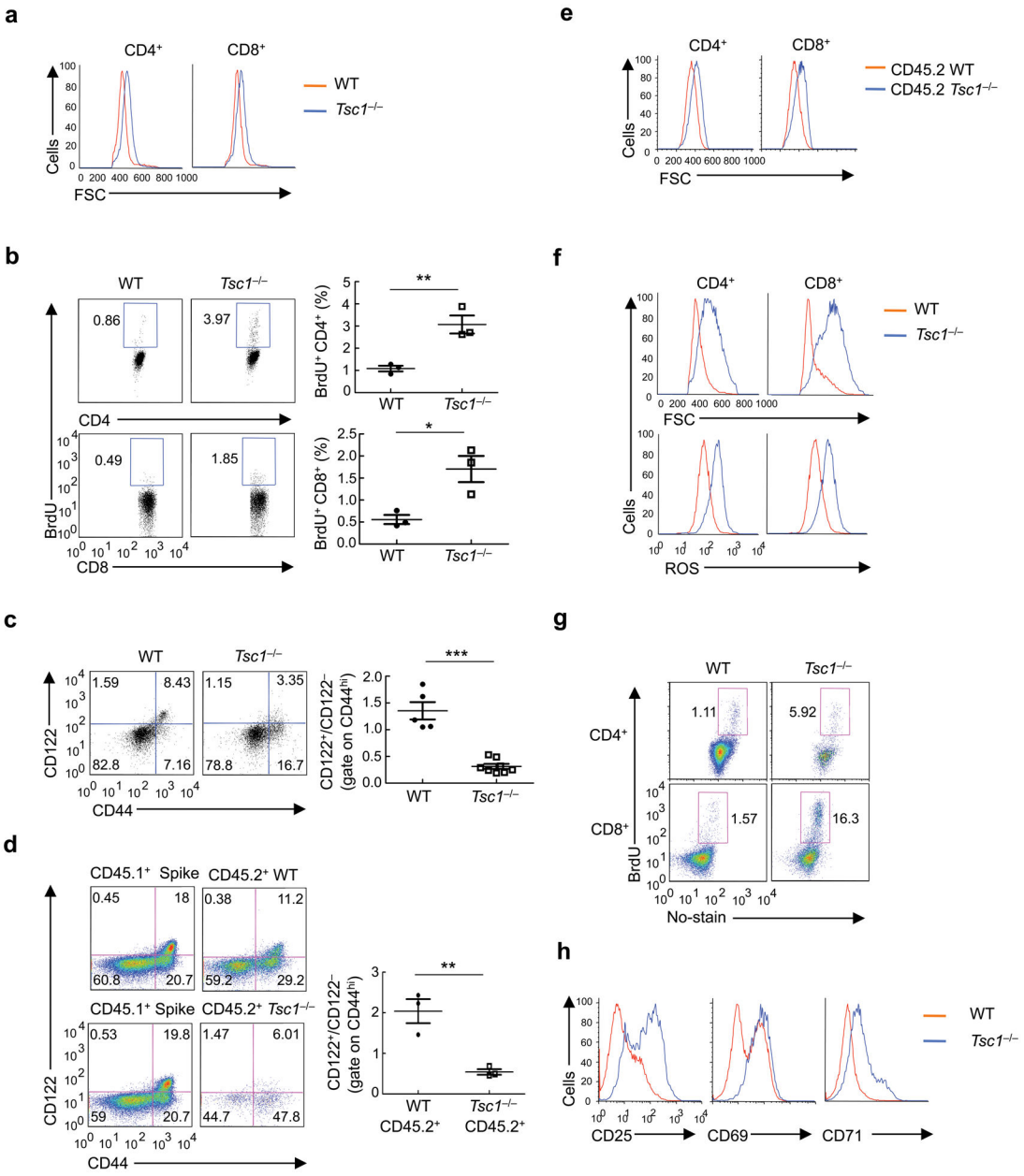

Figure 4. *Tsc1* deficiency causes cell-autonomous loss of quiescence *in vivo* and hyperactive responses to TCR stimulation

(**a**) Cell size of freshly isolated WT and *Tsc1*$^{-/-}$ T cells. FSC, forward scattering. (**b**) BrdU staining in splenocytes of WT and *Tsc1*$^{-/-}$ mice 16 hours after injection with BrdU (n=3). (**c**) CD122 and CD44 expression on gated CD8 splenocytes of WT and *Tsc1*$^{-/-}$ mice. Right, ratios of CD122^{+}/CD122^{-} among CD44^{hi} populations (n=5–8). (**d**) CD122 and CD44 expression on gated CD8 splenocytes in the mixed BM chimeras generated as in Fig. 1e. Right, ratios of CD122^{+}/CD122^{-} cells among CD44^{hi} populations of the CD45.2^{+} cells (n=3). (**e**) Cell size of CD45.2^{+} cells in the mixed BM chimeras. (**f**) Flow cytometry of WT and *Tsc1*$^{-/-}$ naive T cells after stimulation with anti-CD3-CD28 for 16 hours for the measurements of cell size (upper) and ROS production (lower). (**g**) BrdU staining in naive T

cells activated with anti-CD3-CD28 for 20 hours, followed by pulsing with BrdU for 90 min. (**h**) Expression of activation markers in WT and *Tsc1*$^{-/-}$ naive T cells after stimulation with anti-CD3-CD28 for 16 hours. * P <0.05; ** P < 0.01; *** P< 0.0001. Data are representative of 5 (**a,c,f**), 3 (**b,d,e,g**), and 4 (**h**) independent experiments.

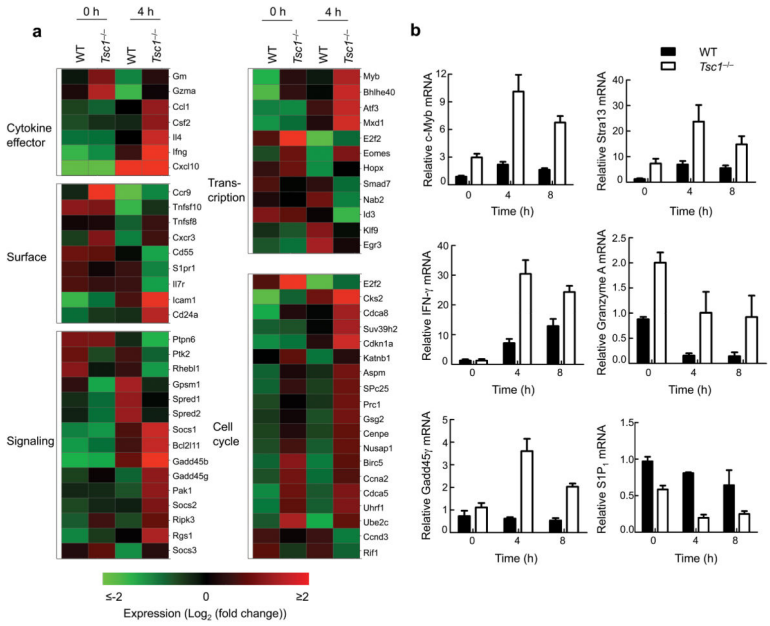

Figure 5. Tsc1-dependent gene expression programs

(**a**) A subset of genes differentially regulated in WT and *Tsc1*$^{-/-}$ CD4 T cells activated with TCR for 4 hours (≥ 2-fold difference with false discovery rate <0.1). Triplicate samples were used in the analysis. (**b**) Real-time PCR analysis of selected genes in WT and *Tsc1*$^{-/-}$ CD4 T cells activated for various times. Data are representative of 2 independent experiments.

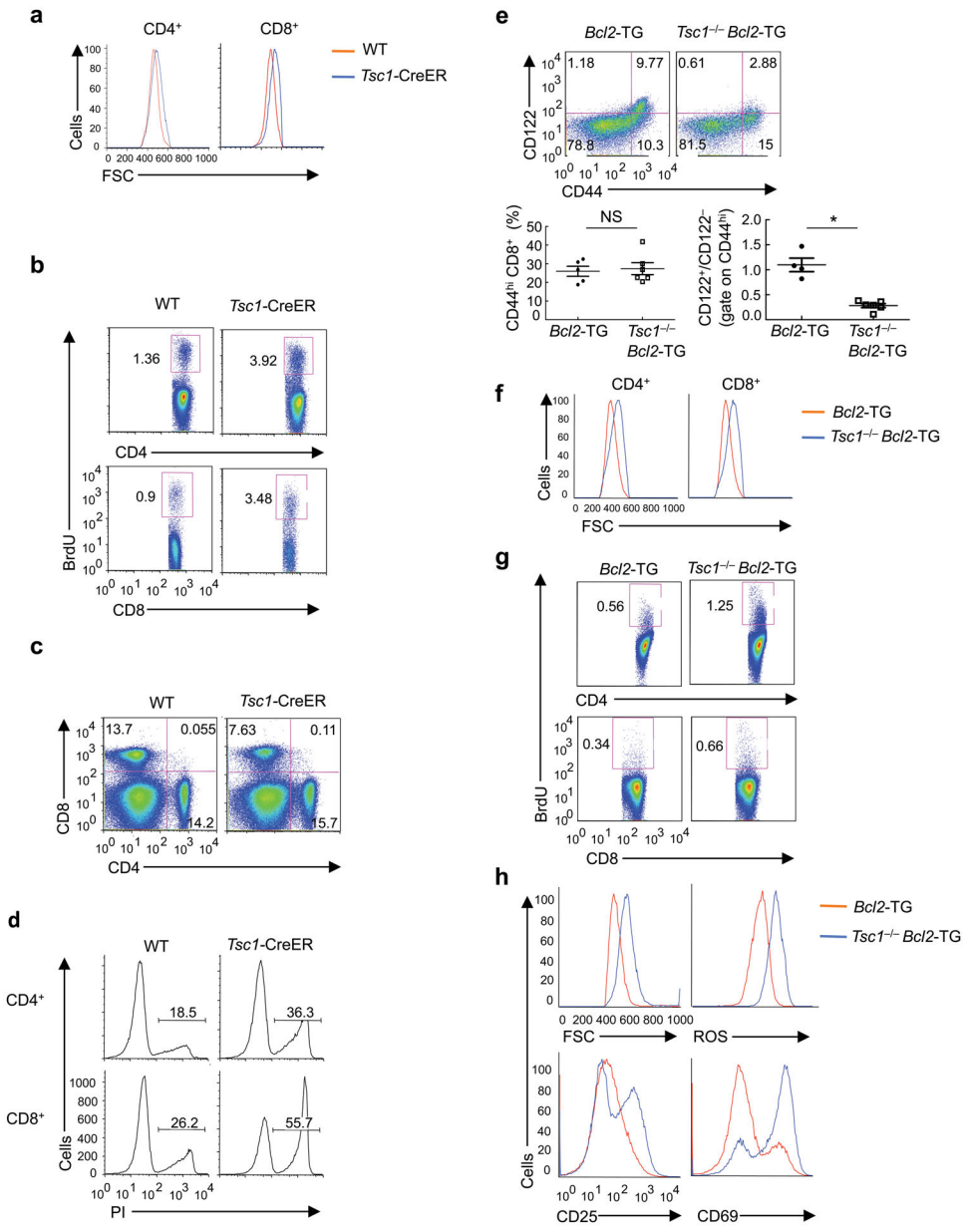

Figure 6. Loss of T cell quiescence results from inducible deletion of *Tsc1* and is independent of cell survival

(**a–d**) Analyses of WT and *Tsc1*-CreER mice at 2 weeks after tamoxifen injection, for cell size of freshly isolated T cells (**a**), BrdU staining in splenocytes of mice 16 hours after injection with BrdU (**b**), splenic T cell populations (**c**), and PI^+ apoptotic cells after 16 hours of anti-CD3-CD28 stimulation of naive T cells (**d**). (**e–h**) Analyses of WT and *Tsc1*$^{-/-}$ mice expressing the *Bcl2* transgene, for expression of CD122 and CD44 on gated CD8 splenocytes (lower left, proportions of total $CD44^{hi}$ populations; lower right, ratios of $CD122^+/CD122^-$ populations among $CD44^{hi}$ cells, n=4–6) (**e**), cell size of freshly isolated T cells (**f**), and BrdU staining in splenocytes of mice 16 hours after injection with BrdU (**g**). (**h**) Flow cytometry of naive T cells after stimulation with anti-CD3-CD28 for 16 hours for

the measurements of cell size, ROS production, and expression of CD25 and CD69. NS, not significant; * $P = 0.0001$. Data are representative of 2 (**a–d,h**), 4 (**e,f**), and 3 (**g**) independent experiments.

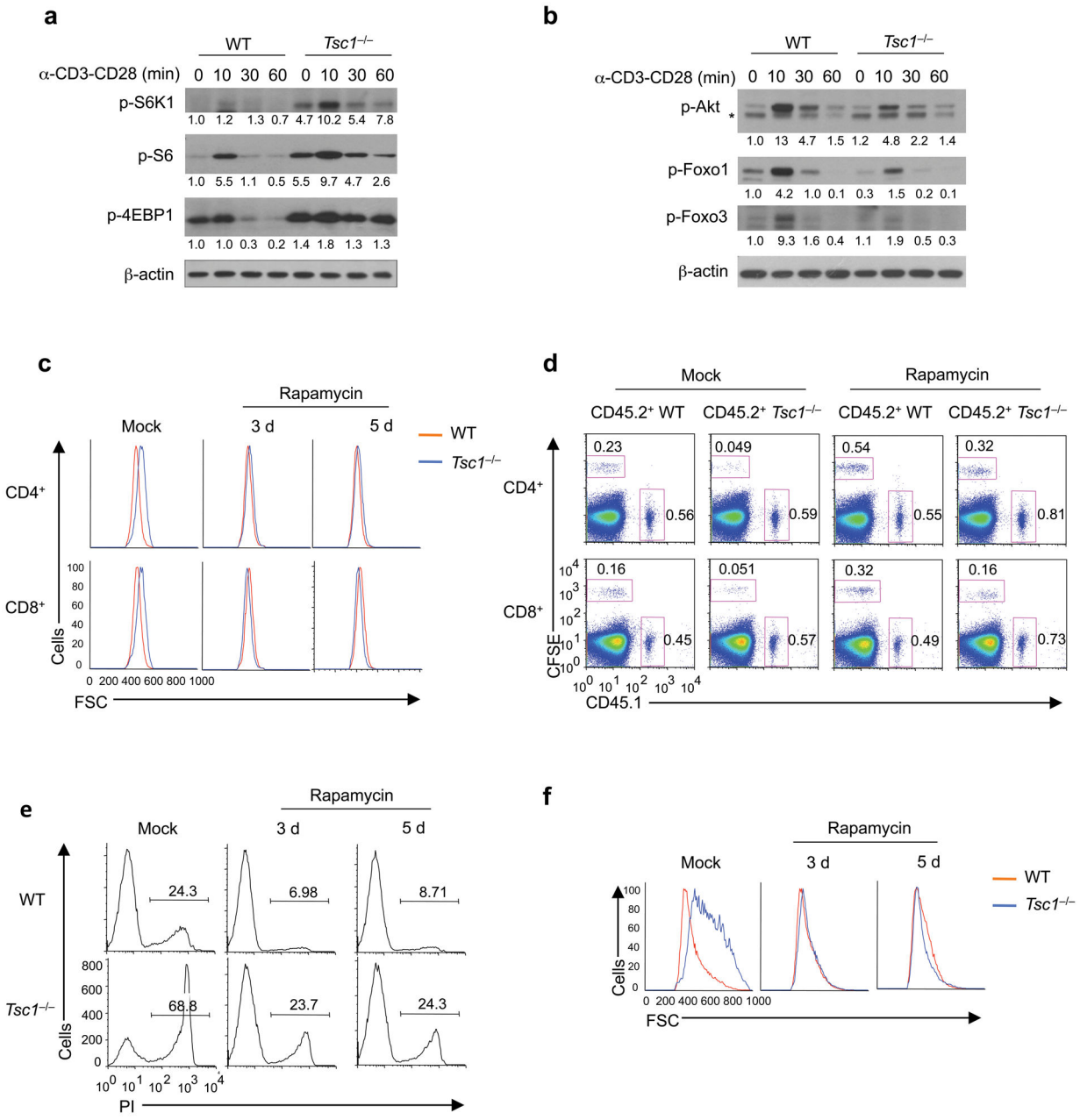

Figure 7. Tsc1 regulates mTORC1 and mTORC2 activities, with mTORC1 activation essential to disrupt immune quiescence and homeostasis

(**a**) Phosphorylation of S6K1, S6 and 4EBP1 in WT and *Tsc1*$^{-/-}$ naive CD4 T cells after they were stimulated with anti-CD3-CD28 for various times. (**b**) Phosphorylation of Akt (Ser473), Foxo1 and Foxo3 in WT and *Tsc1*$^{-/-}$CD4 T cells after they were stimulated with anti-CD3-CD28 for various times. *, non-specific bands. Numbers below lanes (**a,b**) indicate band intensity relative to that of the loading control β-actin. (**c**) Cell size of freshly isolated splenocytes after WT and *Tsc1*$^{-/-}$ mice were treated with daily injection of rapamycin for a total of 3 or 5 days. (**d**) Flow cytometry of splenocytes in the recipient mice (CD45.2^{+}) 6 days after adoptive transfer of equal numbers of CD45.1^{+} (spike) cells and WT

or *Tsc1*$^{-/-}$ donor cells (CFSE-labeled) that were purified from mock or rapamycin-treated mice. Similar results were obtained in the recipient lymph nodes (not shown here). (**e,f**) Naive T cells from rapamycin or mock treated WT and *Tsc1*$^{-/-}$ mice were stimulated with anti-CD3-CD28 overnight, followed by measurements of PI^{+} apoptotic cells (**e**) and cell size (**f**). Data are representative of 5 (**a,b**) and 3 (**c–f**) independent experiments.

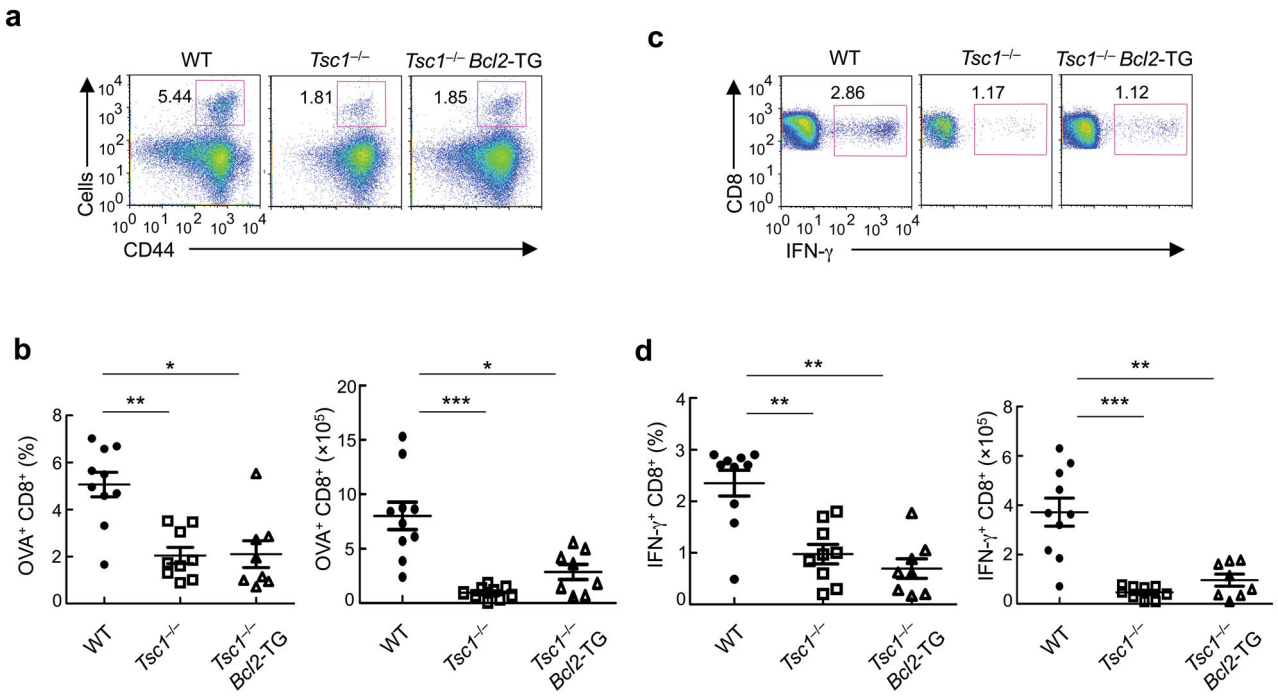

Figure 8. ***Tsc1*** **deficiency dampens antibacterial immune response** ***in vivo***

(**a,b**) Flow cytometry (**a**) and proportions and absolute numbers (**b**) of OVA-reactive tetramer-positive CD8 T cells in WT, *Tsc1*$^{-/-}$ and *Tsc1*$^{-/-}$ *Bcl2*-TG mice infected with LM-OVA. (**c,d**) Flow cytometry (**c**) and proportions and absolute numbers (**d**) of OVA-reactive IFN-γ^{+} CD8 T cells in LM-OVA infected mice, detected after OVA stimulation and intracellular cytokine staining. * P <0.005; ** P < 0.001; *** P< 0.0001. Data are representative of 3 independent experiments (n=8–10).