Tsc1 promotes the differentiation of memory CD8⁺ T cells via orchestrating the transcriptional and metabolic programs

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Memory CD8⁺ T cells are an essential component of protective immunity. Signaling via mechanistic target of rapamycin (mTOR) has been implicated in the regulation of the differentiation of effector and memory T cells. However, little is understood about the mechanisms that control mTOR activity, or the effector pathways regulated by mTOR. We describe here that tuberous sclerosis 1 (Tsc1), a regulator of mTOR signaling, plays a crucial role in promoting the differentiation and function of memory CD8⁺ T cells in response to *Listeria monocytogenes* infection. Mice with specific deletion of Tsc1 in antigen-experienced CD8⁺ T cells evoked normal effector responses, but were markedly impaired in the generation of memory T cells and their recall responses to antigen reexposure in a cell-intrinsic manner. Tsc1 deficiency suppressed the generation of memory-precursor effector cells while promoting short-lived effector cell differentiation. Transcriptome analysis indicated that Tsc1 coordinated gene expression programs underlying immune function, transcriptional regulation, and cell metabolism. Furthermore, Tsc1 deletion led to excessive mTORC1 activity and dysregulated glycolytic and oxidative metabolism in response to IL-15 stimulation. These findings establish a Tsc1-mediated checkpoint in linking immune signaling and cell metabolism to orchestrate memory CD8⁺ T-cell development and function.

T-cell memory | immune response | glycolysis | oxidative phosphorylation

Memory CD8⁺ T cells play an important role in protective immunity with the capability to mount robust recall responses upon reexposure to antigens derived from tumor cells or infection. Recent studies have revealed developmental pathways and transcriptional programs important for the generation of long-lived memory cells (1). Antigen-activated CD8⁺ T cells rapidly expand and generate heterogeneous populations of effector cells, namely short-lived effector cells (SLECs) and memory-precursor effector cells (MPECs) (2, 3). SLECs (CD127^{lo}KLRG1^{hi}) exhibit strong cytotoxicity with high expression of perforin and granzymes and are predisposed to cell death, whereas MPECs (CD127^{hi}KLRG1^{lo}) display the increased potential to survive and further differentiate into mature memory CD8⁺ T cells. The fate decision between SLECs and MPECs is important for effector functions and memory differentiation and is shaped by a number of transcription factors. For instance, transcription factors Eomes and Bcl6 promote the generation of MPECs, whereas Blimp1 and T-bet drive the transcriptional programs for SLEC differentiation (1).

T cells dynamically reprogram cellular metabolism to fulfill the bioenergetics and biosynthetic requirements for their survival, proliferation, and differentiation (4–6). Naïve and memory T cells use catabolic metabolism via oxidative phosphorylation, especially fatty acid oxidation, to produce ATP for their survival. In contrast, antigen-stimulated T cells switch to anabolism to support their rapid proliferation through up-regulating expression of genes involved in multiple metabolic pathways, including glycolysis, fatty acid and cholesterol biosynthesis, and amino acid transport (7–10). Emerging studies indicate that distinct metabolic pathways contribute to the fate decisions of effector and

memory T cells. For instance, the increased glycolytic metabolism promotes effector T-cell generation (11), whereas oxidative phosphorylation and mitochondrial spare respiratory capacity facilitate memory T-cell differentiation (12, 13). Recent studies have also identified transcriptional regulators of cell metabolism that promote effector T-cell differentiation, including HIF1 and IRF4 (14–17). In contrast, how cell metabolism is regulated by immune signaling pathways in effector and memory T-cell differentiation remains unclear.

Mechanistic target of rapamycin (mTOR), an evolutionarily conserved serine–threonine kinase and the catalytic component of mTORC1 and mTORC2 complexes, is a crucial controller of T-cell activation and function (18, 19). mTOR signaling has been implicated in the control of effector and memory T-cell differentiation (20–22). Inhibition of mTOR signaling by rapamycin promotes the generation of MPECs and their subsequent differentiation into memory T cells upon acute lymphocytic choriomeningitis virus (LCMV) infection (20). In vitro treatment of effector cells with rapamycin also enhances the developmental potential of memory cells through increasing the expression of Eomes at the expense of T-bet (21). Moreover, rapamycin enhances the ability of homeostatic proliferation-induced memory CD8⁺ T cells against tumor challenge via regulating the expression of Eomes and T-bet (22). Despite these studies of linking mTOR signaling to the regulation of memory T-cell differentiation, the upstream regulators of mTOR remain unresolved. Notably, deletion of Pten,

Significance

Enhancing the generation and function of memory T cells represents a crucial strategy to improve protective immunity against pathogens and tumors. The signaling pathway via mechanistic target of rapamycin (mTOR) has been implicated in the regulation of the differentiation of effector and memory T cells, but the upstream regulators or downstream mechanisms remain unclear. In this study, we provide insight into the mechanistic basis that controls mTOR signaling and memory T-cell responses. The deficiency of tuberous sclerosis 1 (Tsc1) in antigen-experienced T cells impairs the differentiation of memory T-cell precursors and the formation of memory T cells, associated with excessive mTOR activity and dysregulated cell metabolism. Our study establishes a molecular mechanism that links mTOR signaling and cell metabolism for memory T-cell development.

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significantly reduced in *Tsc1*^{-/-} mice (Fig. 2A). We next assessed cytokine production of WT and *Tsc1*^{-/-} splenocytes after restimulation with the OVA peptide ex vivo. *Tsc1*^{-/-} mice had reduced frequency and number of T cells producing IFN- γ and TNF- α compared with WT mice (Fig. 2B). Therefore, *Tsc1* deficiency impairs the recall response of memory cells.

One potential caveat of the experimental system above was that the defective recall response of *Tsc1*^{-/-} memory T cells could be ascribed to the uneven numbers of memory T cells in WT and *Tsc1*^{-/-} mice before rechallenge. To circumvent this possibility, we sorted OVA-specific memory T cells from WT and *Tsc1*^{-/-} mice (CD45.2⁺) at day 41 p.i. and adoptively transferred equal numbers of WT and *Tsc1*^{-/-} memory T cells into naïve recipients (CD45.1⁺), followed by LM-OVA infection 24 h later. At day 5 p.i., we assessed the frequency of donor-derived cells in various organs. The frequency of *Tsc1*^{-/-} donor cells was significantly reduced compared with that of WT donor cells in all organs examined (Fig. 2C). We conclude that *Tsc1* is required for the recall response of memory cells upon antigen reexposure.

A Cell-Intrinsic Requirement of *Tsc1* in Memory Formation and Function.

Given the role of *Tsc1* in the homeostasis of naïve T cells (27–29), it remains possible that the T-cell receptor (TCR) repertoire of *Tsc1*^{-/-} T cells could be altered and contributed to the defects observed above. We therefore crossed *Tsc1*^{-/-} mice onto the TCR-transgenic background (OT-I) in which CD8⁺ T cells expressed a SIINFEKL peptide-specific TCR. We mixed congenically marked naïve OT-I and *Tsc1*^{-/-}OT-I cells at a 1:1 ratio and cotransferred them to WT recipient mice, followed by LM-OVA infection. This adoptive transfer system also allowed us to exclude the effects of potential *Tsc1* deletion in cells other than CD8⁺ T cells. Consistent with the normal effector responses observed in *Tsc1*^{-/-} mice (Fig. S2), the frequencies of OT-I and *Tsc1*^{-/-}OT-I

cells were comparable at day 9 p.i. (Fig. 3A). However, at day 29 and 57 p.i., the percentage of *Tsc1*^{-/-}OT-I cells was substantially lower than that of OT-I cells (Fig. 3A), indicative of a defect in the generation of memory cells. Upon LM-OVA rechallenge, the frequencies of *Tsc1*^{-/-}OT-I cells were also significantly lower than those of OT-I cells in various organs examined (Fig. 3B). Moreover, among IFN- γ - or TNF- α -producing cells in the recipient mice, the majority of them were derived from OT-I instead of *Tsc1*^{-/-}OT-I donor cells (Fig. 3C and D). These results establish a cell-intrinsic role of *Tsc1* in promoting the formation and function of memory CD8⁺ T cells.

Impaired Differentiation of Memory Precursors in the Absence of *Tsc1*.

Aside from rapid expansion, effector cells differentiate into diverse subsets with distinct potentials for the formation of memory T cells: MPECs (CD127^{hi}KLGR1^{lo}) and SLECs (CD127^{lo}KLGR1^{hi}) (2, 3). To investigate whether *Tsc1* deficiency affects the differentiation of MPECs and SLECs, we examined the expression of CD127 and KLGR1 on WT and *Tsc1*^{-/-} effector cells at day 9 p.i. We found that *Tsc1*^{-/-} OVA-specific CD8⁺ T cells had markedly reduced frequency and number of MPECs, whereas the frequency and number of SLECs were increased (Fig. 4A). Similar findings were observed in *Tsc1*^{-/-}OT-I T cells in the adoptive transfer system (Fig. S4A). Moreover, to exclude the potential role of *Tsc1* in cell survival (Fig. S3A), we crossed *Tsc1*^{-/-} mice with mice expressing a Bcl2 transgene in lymphocytes (*Bcl2*-transgenic, *Bcl2*-Tg) (29). Following LM-OVA infection, *Tsc1*^{-/-}Bcl2-Tg T cells showed reduced MPECs but increased SLECs, compared with the Bcl2-Tg counterparts (Fig. S4B). Taken together, these results reveal an intrinsic effect of *Tsc1* deficiency on the fate decisions between MPECs and SLECs.

The differentiation of activated CD8⁺ T cells into MPECs and SLECs is controlled by the differential expression of specific transcription factors, with Eomes and Bcl6 important in promoting the differentiation of MPECs (1). The expression of these transcription factors in antigen-specific CD8⁺ T cells from WT and *Tsc1*^{-/-} mice at day 9 p.i. was largely comparable (Fig. S5). However, *Tsc1* deficiency considerably enhanced the expression of Blimp1 and T-bet, which are crucial factors for the development of SLECs and cytotoxic T-lymphocyte (CTL) functions (1) (Fig. 4B). The altered expression of Blimp1 and T-bet was observed in subdivided populations including MPECs, SLECs, and CD127⁺KLGR1⁺ cells (Fig. 4C), highlighting a direct effect of *Tsc1* deficiency on these transcription factors. Moreover, *Tsc1*^{-/-} CD8⁺ T cells had elevated expression of Granzyme B and immune inhibitory receptors CD244 (2B4), Tim-3, and CTLA-4 (32–34) (Fig. 4D). Therefore, consistent with the impaired memory development and MPEC formation, *Tsc1* deficiency enhances the expression of effector-promoting transcription factors Blimp1 and T-bet and multiple immune inhibitory receptors.

Tsc1-Dependent Gene Expression Programs in Antigen-Specific CD8⁺ T Cells.

We performed functional genomics to identify additional pathways controlled by *Tsc1* by comparing global gene expression profiles of WT and *Tsc1*^{-/-} OVA-specific CD8⁺ T cells sorted from mice at day 9 p.i. *Tsc1*^{-/-} cells contained a total of 420 increased probes and 378 decreased probes by greater than 0.5 log₂ fold change between the comparison (Fig. 5A). The heat maps in Fig. 5B showed that *Tsc1*^{-/-} CD8⁺ T cells differentially expressed multiple genes (with log₂ fold change > 0.5) associated with the differentiation of effector and memory CD8⁺ T cells, including receptors and secreted factors (*Il12rb2*, *Gzmb*, *Sell*, and *Il7r*) and transcription factors (*Tcf7*, *Bach2*, and *Klf4*). Additionally, *Tsc1*^{-/-} CD8⁺ T cells had altered expression of genes involved in various metabolic pathways, including glycolysis (*Tpi1*), cholesterol synthesis (*Fdps*, *Hmgcs1*, and *Insig1*), and fatty acid synthesis (*Fads1* and *Elovl7*) (Fig. 5B). Therefore, *Tsc1* coordinates the expression of immune response and metabolic genes in antigen-specific CD8⁺ T cells.

Given the effects of *Tsc1* on the differentiation of MPECs and SLECs, it remains possible that the altered gene profiles of

Fig. 2. *Tsc1* deficiency impairs the recall response of memory CD8⁺ T cells. (A) WT and *Tsc1*^{-/-} mice were infected with LM-OVA and rechallenged with LM-OVA 35 d later. The recall response of memory CD8⁺ T cells was examined at day 4 after secondary infection. Shown are representative flow cytometry plots of CD8⁺ T cells (Left) and the frequency (Center) and number (Right) of K^b-OVA⁺ CD8⁺ T cells. (B) At day 4 after the secondary infection, splenocytes from WT and *Tsc1*^{-/-} mice were isolated and restimulated with the SIINFEKL peptide for intracellular cytokine staining of IFN- γ and TNF- α . Shown are representative flow cytometry plots of CD8⁺ T cells (Left) and the frequency (Center) and number (Right) of IFN- γ ⁺ and TNF- α ⁺ CD8⁺ T cells. (C) Memory CD8⁺ T cells (CD45.2⁺) sorted from WT and *Tsc1*^{-/-} mice at day 41 p.i. were transferred into naïve CD45.1⁺ recipients. At 24 h, the recipients were challenged with LM-OVA and analyzed 5 d later for CD45.1 and CD45.2 staining of CD8⁺ T cells (Left) and the frequency of CD45.2⁺ donor cells (Right). BM, bone marrow; PLN, peripheral lymph nodes. Data are representative of two independent experiments and are presented as the mean $\pm$ SEM.

Fig. 3. *Tsc1* promotes memory T-cell generation through cell-intrinsic mechanisms. Naïve CD8⁺ T cells from OT-I⁺ (CD45.2⁺) and *Tsc1*^{-/-} OT-I⁺ (CD45.12⁺) mice were isolated, mixed at a 1:1 ratio, and transferred to CD45.1⁺ recipients, followed by LM-OVA infection 1 d later. (A) Flow cytometry and frequencies of OT-I⁺ (CD45.2⁺) and *Tsc1*^{-/-} OT-I⁺ (CD45.12⁺) donor T cells out of the total K^b-OVA⁺ CD8⁺ T cells in the blood at days 9, 29, and 57 p.i., mice were rechallenged with LM-OVA and analyzed 3 d later by flow cytometry. (Right) The frequencies and numbers of OT-I⁺ (CD45.2⁺) and *Tsc1*^{-/-} OT-I⁺ (CD45.12⁺) out of the total K^b-OVA⁺ CD8⁺ T cells from the blood, spleen, and liver (B) or out of splenic IFN- γ ⁺ (C) or TNF- α ⁺ (D) CD8⁺ T cells after restimulation with SIINFEKL peptide. Data are representative of three independent experiments and are presented as the mean $\pm$ SEM.

Tsc1^{-/-} cells were simply secondary to the disrupted ratios of MPECs and SLECs. To address this issue, we compared *Tsc1*-dependent targets with the genes differentially expressed in KLRG1^{hi} and KLRG1^{lo} CD8⁺ T cells in the public database (35). Out of 798 probes with a log₂ fold change > 0.5 in *Tsc1*^{-/-} cells, 621 were matched to this database and were included for further analysis (Dataset S1). These *Tsc1* targets were partitioned into four distinct clusters that differed in their relationship to the specific gene targets in KLRG1^{hi} cells (Fig. 5C). A salient feature was that the majority of *Tsc1* targets (408 out of 621 probes) fell into cluster 1 (red circles), in which their expression was comparable between KLRG1^{hi} and KLRG1^{lo} cells (<0.5 log₂ fold change). Further, cluster 2 (blue circles) contained 38 probes that showed the opposite direction of change in expression, and cluster 3 (green circles) contained 49 probes that were differentially expressed in both types of comparisons, but to a greater extent in KLRG1^{hi} cells compared with *Tsc1*^{-/-} cells. Only cluster 4 (yellow circles, 126 probes) contained the target genes with equal magnitude of change (>0.5 log₂ fold change) in both *Tsc1*^{-/-} and KLRG1^{hi} cells, thus representing concordant changes in both types of cells. Overall, ~80% of all *Tsc1* gene targets are independent of KLRG1 expression (clusters 1–3), indicating a distinct gene expression program controlled by *Tsc1*.

We next used the ingenuity pathway analysis (IPA) system to analyze canonical pathways controlled by *Tsc1* in activated CD8⁺ T cells by investigating the differentially expressed genes with a false discovery rate (FDR) < 0.1. Fig. S6A shows the top 11 canonical pathways affected by *Tsc1* deficiency with *P* < 0.01, including oxidative phosphorylation and mitochondria dysfunction. Finally, to identify key networks regulated by *Tsc1*, we did a gene-set enrichment analysis (GSEA) to compare the gene expression profiles of WT and *Tsc1*^{-/-} K^b-OVA⁺ CD8⁺ cells. This unbiased approach identified multiple pathways with significant enrichment, including cell cycle, cholesterol biosynthesis, and oxidative phosphorylation, all of which were up-regulated in *Tsc1*^{-/-} CD8⁺ T cells (Fig. S6 B–D).

Given that oxidative phosphorylation was affected by *Tsc1* deficiency in both GSEA and IPA analysis, we examined mitochondrial reactive oxygen species (ROS) production, which is associated with oxidative phosphorylation (5). *Tsc1*^{-/-} CD8⁺ T cells showed increased ROS production at day 9 and 18 p.i. (Fig. 5D). Moreover, *Tsc1*^{-/-} CD8⁺ cells had larger cell sizes than WT cells (Fig. 5E),

indicative of increased cell growth. Altogether, these data indicate that *Tsc1* coordinates diverse pathways involved in immune function, transcriptional regulation, and cell growth and metabolism.

Dysregulated mTORC1 and Metabolic Activities in *Tsc1*-Deficient CD8⁺ T Cells. Cytokines IL-2 and IL-15 are critical for the development and maintenance of CD8⁺ effector and memory T cells, respectively (36). To understand upstream and downstream mechanisms underlying *Tsc1*-dependent memory T-cell differentiation, we used an in vitro culture system that mimics the programs of effector and memory T-cell differentiation (13, 37) and examined the effect of acute deletion of *Tsc1* in this system. To this end, we crossed *Tsc1*^{fl/fl} with *Rosa26-Cre-ER*^{T2} (CreER) and OT-I transgenic mice. We sorted naïve CD8⁺ T cells from *Tsc1*^{+/+} CreER⁺ OT-I and *Tsc1*^{fl/fl} CreER⁺ OT-I mice and activated them with OVA and IL-2 in the presence of 4-hydroxytamoxifen (4-OHT) for 4 d, followed by an additional culture in either IL-2 or IL-15. The deletion of *Tsc1* after 4-OHT treatment was confirmed by real-time PCR analysis (Fig. S7A), which was also reflected in the increased cell size (Fig. S7B). IL-2 and IL-15 induce distinct effects on T-cell growth and metabolic activities (13, 38), although how these metabolic programs are regulated is poorly defined. We therefore examined oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which denote mitochondria respiration and glycolysis, respectively, in cells cultured with IL-2 or IL-15. Consistent with previous observations (13), IL-15 mediated a stronger effect on OCR than IL-2 did, whereas IL-2 but not IL-15 potently elevated ECAR activity (Fig. 6A and B). Importantly, *Tsc1* deficiency markedly enhanced ECAR and OCR in cells cultured with IL-15, but effects were very modest in cells cultured with IL-2 (Fig. 6A and B). Thus, *Tsc1* deletion results in dysregulated metabolic activity during IL-15-mediated differentiation of memory T cells.

Moreover, compared with *Tsc1*-sufficient controls, *Tsc1*-deficient T cells up-regulated mTORC1 activity, as indicated by the increased phosphorylation of the ribosomal protein S6. Notably, the extent of up-regulation over control cells was much more profound in IL-15-stimulated conditions (~7.5-fold) compared with IL-2-stimulated conditions (~2.5-fold) (Fig. 6C). Furthermore, treatment of IL-15-stimulated cells with rapamycin considerably

Fig. 4. *Tsc1* is required for the differentiation of MPECs. (A) Flow cytometry of CD127 and KLRG1 (Top) on K^b-OVA⁺ CD8⁺ T cells from WT and *Tsc1*^{-/-} mice at day 9 p.i., and the frequency (Middle) and number (Bottom) of MPECs (CD127^{hi}KLRG1^{lo}) and SLECs (CD127^{lo}KLRG1^{hi}). (B) Intracellular staining of Blimp1 and T-bet in splenic K^b-OVA⁺ CD8⁺ T cells from WT and *Tsc1*^{-/-} mice at day 9 p.i. (C) Intracellular staining of Blimp1 and T-bet in MPECs, SLECs, and CD127^{hi}KLRG1^{hi} cells in splenic K^b-OVA⁺ CD8⁺ T cells from WT and *Tsc1*^{-/-} mice at day 9 p.i. (D) Flow cytometry of GzmB, CD244, Tim-3, and CTLA-4 at day 9 p.i. in K^b-OVA⁺ CD8⁺ T cells from WT and *Tsc1*^{-/-} mice. Mean fluorescent intensity (MFI) is presented above the plots (WT, red; *Tsc1*^{-/-}, blue; B–D). Data are representative of three independent experiments and are presented as the mean $\pm$ SEM.

Fig. 5. Tsc1-dependent gene expression profiles in antigen-experienced CD8⁺ T cells. (A) Scatterplot comparison of global gene expression profiles between WT and *Tsc1*^{-/-} K^b-OVA⁺ CD8⁺ T cells sorted from mice at day 9 p.i.; transcripts with >0.5 log₂ fold change are highlighted (WT, *n* = 4; *Tsc1*^{-/-}, *n* = 5). (B) Heat maps of differentially expressed genes in K^b-OVA⁺ CD8⁺ T cells (with >0.5 log₂ fold change), with red color denoting up-regulated genes in *Tsc1*^{-/-} cells and blue color down-regulated genes in *Tsc1*^{-/-} cells. (C) Comparison of expression changes in *Tsc1*^{-/-} versus WT cells with those in KLRG1^{hi} versus KLRG1^{lo} cells. The Tsc1 target genes (>0.5 log₂ fold change) were partitioned into four main clusters, shown and colored by regions (R1–R4). Numbers within parentheses indicate the number of probes within each subregion. (D and E) ROS production (D) and cell size (E) of splenic K^b-OVA⁺ CD8⁺ T cells from WT and *Tsc1*^{-/-} mice at day 9 and 18 p.i. Data are representative of one experiment (A–C) and two independent experiments (D and E) and are presented as the mean ± SEM.

blocked the dysregulated mTORC1 (Fig. 6D), OCR (Fig. 6E), and ECAR activities (Fig. 6F), as well as T-cell glycolytic activity (Fig. 6G). Therefore, excessive mTORC1 activation in the absence of Tsc1 drives the metabolic dysregulation. Moreover, compared with IL-2, IL-15 has more of a stringent requirement for Tsc1 functions in actively suppressing mTORC1 and mTORC1-dependent metabolic activities.

Associated with dysregulated metabolism, Tsc1-deficient cells down-regulated CD62L and Bcl2 expression (Fig. 6G) but up-regulated Caspase-3 activity (Fig. 6H). Importantly, rapamycin treatment restored the excessive activity of mTORC1 and the dysregulated expression of CD62L, Bcl2, and Caspase-3 activity (Fig. 6G and H). Therefore, Tsc1 links mTORC1 activation, cell metabolism, and immune regulation.

Discussion

In this study, we reveal an important function of Tsc1 in promoting the differentiation and function of memory CD8⁺ T cells in bacterial infection. By developing a mouse model to ablate Tsc1 specifically in antigen-experienced CD8⁺ T cells, we found that Tsc1 is dispensable for effector responses at the expansion phase, but is essential for the formation of memory CD8⁺ T cells and their recall responses to antigen reexposure in a T-cell-intrinsic manner. Mechanistically, deficiency of Tsc1 dampens the differentiation of MPECs, associated with the impaired transcriptional and metabolic programs. In particular, loss of Tsc1 results in excessive mTORC1 activity and dysregulated glycolytic and oxidative metabolism in response to IL-15 stimulation. These findings provide new mechanistic insight into T-cell memory formation and function and highlight that modulation of Tsc1 function is a potential strategy to improve the quality and quantity of memory responses.

Emerging studies highlight a pivotal role of mTOR signaling in the fate decisions of effector and memory T cells (20–23), but the upstream regulators of mTOR remain unresolved. Notably, mTOR can be activated by Akt-independent pathways in effector CD8⁺ T cells (14, 25), and deletion of Pten does not cause significant defects in memory formation (23). Here we circumvented the requirements of mTOR signaling in naive T-cell homeostasis (27–29) and immediate TCR activation (10), by specific ablation of Tsc1 in antigen-experienced CD8⁺ T cells. Although Tsc1 deficiency elevates mTORC1 activity, this does not significantly impact the generation of effector CD8⁺ T cells

at the peak phase of primary responses. Instead, Tsc1 function is essential to ensure proper differentiation between MPECs and SLECs, which is a prerequisite for the subsequent generation of memory T cells (1). Specifically, Tsc1 deficiency suppresses the formation of MPECs and reciprocally promotes the formation of SLECs. These effects were observed in multiple independent systems including direct challenge, adoptive transfer of OT-I cells, and *Bcl2*-Tg backgrounds, thereby highlighting a direct role

Fig. 6. Tsc1 deficiency leads to dysregulated mTORC1 activity and metabolism in activated CD8⁺ T cells. (A and B) Naive CD8⁺ T cells from OT-I⁺ *Tsc1*^{fl/fl} CreER⁺ (WT) and OT-I⁺ *Tsc1*^{fl/fl} CreER⁺ (*Tsc1*^{CreER}) mice were stimulated with OVA in the presence of IL-2 and 4-OHT for 4 d, and live cells were isolated and cultured with IL-15 or IL-2 for 5 d. OCR and ECAR were measured in IL-15– (A) and IL-2–cultured cells (B). (C) Phosphorylation of S6 in WT and *Tsc1*^{CreER} cells activated with OVA and then stimulated with IL-2 or IL-15 for 18 h. Numbers below lanes indicate band intensity relative to that of β-actin (loading control). (D) Flow cytometry of p-S6 phosphorylation in WT and *Tsc1*^{CreER} cells activated with OVA and then stimulated with IL-15 for 1 and 2 d in the absence or presence of rapamycin (Rapa). (E and F) OCR (E) and ECAR (F) of WT and *Tsc1*^{CreER} cells activated with OVA and then stimulated with IL-15 for 24 h in the absence or presence of rapamycin. (G and H) Flow cytometry of CD62L, Bcl2 (G), and Caspase-3 activity (H) in WT and *Tsc1*^{CreER} cells activated with OVA and then stimulated with IL-15 for 2–3 d in the absence or presence of rapamycin. Data are representative of three independent experiments and are presented as the mean ± SEM.

of Tsc1 in cell fate decisions. Accordingly, Tsc1-deficient antigen-specific CD8⁺ T cells have increased expression of Blimp1 and T-bet, which likely suppress the function of transcription factors required for memory differentiation (1). Additionally, Tsc1 deletion up-regulates inhibitory receptors associated with T-cell exhaustion (32–34). Moreover, Tsc1 deficiency impairs the expression of genes involved in T-cell trafficking, such as L-selectin (*Sell*) and CCR7, which are required for the migration to central-memory T cells in the T-cell areas of secondary lymphoid organs (24). These results indicate that Tsc1 promotes memory T-cell responses, in part, by impinging upon the transcriptional programs required for memory precursor differentiation.

The metabolic programs are dynamically regulated to match the differentiation and function of T cells (4–6). For the fate decisions between effector and memory CD8⁺ T cells, the glycolytic and lipid synthetic metabolism promotes effector T-cell generation (7, 11), whereas oxidative phosphorylation and mitochondrial activity facilitate memory development (12, 13). From the microarray and bioinformatics analyses, we found that *Tsc1*^{−/−} antigen-specific CD8⁺ T cells exhibit elevated expression of metabolic genes involved in glycolysis, lipid synthesis, and oxidative phosphorylation. Moreover, Tsc1-deficient T cells show enhanced glycolytic and oxidative phosphorylation rates in vitro that are closely linked with IL-15-mediated signaling. Although the increased glycolysis upon Tsc1 deletion is in agreement with impaired development of memory cells (11), the effect of the increased oxidative phosphorylation remains to be established. Notably, the IPA analysis indicates that Tsc1 deficiency impinges upon both oxidative phosphorylation and mitochondria function. Further, loss of Tsc1 results in excessive production of mitochondrial ROS. These findings indicate that Tsc1 functions to ensure proper regulation of the metabolic programs, the disruption of which may contribute to impaired memory T-cell differentiation.

We and others have previously established a key role of Tsc1 in the establishment of naïve T-cell quiescence (27–29). Although the exit from quiescence in response to TCR stimulation is essential for proper T-cell activation and effector responses (10), how memory T cells reestablish a quiescent state is unclear. The increased cell size and dysregulated metabolic activities of Tsc1-deficient T cells are consistent with an impaired quiescence state of memory T cells. Additionally, gene expression profiling indicated that Tsc1-deficient CD8⁺ T cells down-regulate the expression of transcription factors required for the maintenance of cell quiescence including Bach2 and Klf4 (39–41). Therefore, the failure to reestablish quiescence in Tsc1-deficient memory T cells is the likely basis for their poor recall responses to antigen rechallenge. This is reminiscent of the observation that abrogated quiescence of naïve T cells is associated with the defective initiation of primary immune responses (29).

In summary, Tsc1 functions as a critical controller of the generation and function of memory T cells. Tsc1 regulates the formation of memory T cells through shaping the fate decisions between MPECs and SLECs and by orchestrating the transcriptional and metabolic programs. Modulation of Tsc1 functions in T cells represents a potential strategy to enhance the quantity and quality of memory T cells against infections and tumors.

Materials and Methods

Mice were purchased from the Jackson Laboratory or described previously. All mice have been extensively backcrossed to the C57BL/6 background. Details of experimental procedures and reagents can be found in *SI Materials and Methods*.

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- Kaech SM, Cui W (2012) Transcriptional control of effector and memory CD8⁺ T cell differentiation. *Nat Rev Immunol* 12(11):749–761.
- Joshi NS, et al. (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27(2):281–295.
- Sarkar S, et al. (2008) Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205(3):625–640.
- Wang R, Green DR (2012) Metabolic checkpoints in activated T cells. *Nat Immunol* 13(10):907–915.
- Pearce EL, Poffenberger MC, Chang CH, Jones RG (2013) Fueling immunity: Insights into metabolism and lymphocyte function. *Science* 342(6155):1242–1245.
- MacIver NJ, Michalek RD, Rathmell JC (2013) Metabolic regulation of T lymphocytes. *Annu Rev Immunol* 31:259–283.
- Kidani Y, et al. (2013) Sterol regulatory element-binding proteins are essential for the metabolic programming of effector T cells and adaptive immunity. *Nat Immunol* 14(5):489–499.
- Sindair LV, et al. (2013) Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. *Nat Immunol* 14(5):500–508.
- Wang R, et al. (2011) The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. *Immunity* 35(6):871–882.
- Yang K, et al. (2013) T cell exit from quiescence and differentiation into Th2 cells depend on Raptor-mTORC1-mediated metabolic reprogramming. *Immunity* 39(6):1043–1056.
- Sukumar M, et al. (2013) Inhibiting glycolytic metabolism enhances CD8⁺ T cell memory and antitumor function. *J Clin Invest* 123(10):4479–4488.
- Pearce EL, et al. (2009) Enhancing CD8 T-cell memory by modulating fatty acid metabolism. *Nature* 460(7251):103–107.
- van der Windt GJ, et al. (2012) Mitochondrial respiratory capacity is a critical regulator of CD8⁺ T cell memory development. *Immunity* 36(1):68–78.
- Finlay DK, et al. (2012) PDK1 regulation of mTOR and hypoxia-inducible factor 1 integrate metabolism and migration of CD8⁺ T cells. *J Exp Med* 209(13):2441–2453.
- Man K, et al. (2013) The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nat Immunol* 14(11):1155–1165.
- Doedens AL, et al. (2013) Hypoxia-inducible factors enhance the effector responses of CD8(+) T cells to persistent antigen. *Nat Immunol* 14(11):1173–1182.
- Yao S, et al. (2013) Interferon regulatory factor 4 sustains CD8(+) T cell expansion and effector differentiation. *Immunity* 39(5):833–845.
- Araki K, Youngblood B, Ahmed R (2010) The role of mTOR in memory CD8 T-cell differentiation. *Immunol Rev* 235(1):234–243.
- Chi H (2012) Regulation and function of mTOR signalling in T cell fate decisions. *Nat Rev Immunol* 12(5):325–338.
- Araki K, et al. (2009) mTOR regulates memory CD8 T-cell differentiation. *Nature* 460(7251):108–112.
- Rao RR, Li Q, Odunsi K, Shrikant PA (2010) The mTOR kinase determines effector versus memory CD8⁺ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin. *Immunity* 32(1):67–78.
- Li Q, et al. (2011) A central role for mTOR kinase in homeostatic proliferation induced CD8⁺ T cell memory and tumor immunity. *Immunity* 34(4):541–553.
- Hand TW, et al. (2010) Differential effects of STAT5 and PI3K/AKT signaling on effector and memory CD8 T-cell survival. *Proc Natl Acad Sci USA* 107(38):16601–16606.
- Finlay D, Cantrell DA (2011) Metabolism, migration and memory in cytotoxic T cells. *Nat Rev Immunol* 11(2):109–117.
- Macintyre AN, et al. (2011) Protein kinase B controls transcriptional programs that direct cytotoxic T cell but is dispensable for T cell metabolism. *Immunity* 34(2):224–236.
- Laplanche M, Sabatini DM (2012) mTOR signaling in growth control and disease. *Cell* 149(2):274–293.
- O'Brien TF, et al. (2011) Regulation of T-cell survival and mitochondrial homeostasis by TSC1. *Eur J Immunol* 41(11):3361–3370.
- Wu Q, et al. (2011) The tuberous sclerosis complex-mammalian target of rapamycin pathway maintains the quiescence and survival of naïve T cells. *J Immunol* 187(3):1106–1112.
- Yang K, Neale G, Green DR, He W, Chi H (2011) The tumor suppressor Tsc1 enforces quiescence of naïve T cells to promote immune homeostasis and function. *Nat Immunol* 12(9):888–897.
- Xie DL, Wu J, Lou YL, Zhong XP (2012) Tumor suppressor TSC1 is critical for T-cell anergy. *Proc Natl Acad Sci USA* 109(35):14152–14157.
- Jacob J, Baltimore D (1999) Modelling T-cell memory by genetic marking of memory T cells in vivo. *Nature* 399(6736):593–597.
- Blackburn SD, et al. (2009) Coregulation of CD8⁺ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 10(1):29–37.
- Jin HT, et al. (2010) Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA* 107(33):14733–14738.
- Wherry EJ (2011) T cell exhaustion. *Nat Immunol* 12(6):492–499.
- Hess Michelini R, Doedens AL, Goldrath AV, Hedrick SM (2013) Differentiation of CD8 memory T cells depends on Foxo1. *J Exp Med* 210(6):1189–1200.
- Mitchell DM, Ravkov EV, Williams MA (2010) Distinct roles for IL-2 and IL-15 in the differentiation and survival of CD8⁺ effector and memory T cells. *J Immunol* 184(12):6719–6730.
- Weninger W, Crowley MA, Manjunath N, von Andrian UH (2001) Migratory properties of naïve, effector, and memory CD8(+) T cells. *J Exp Med* 194(7):953–966.
- Cornish GH, Sinclair LV, Cantrell DA (2006) Differential regulation of T-cell growth by IL-2 and IL-15. *Blood* 108(2):600–608.
- Tsukumo S, et al. (2013) Bach2 maintains T cells in a naïve state by suppressing effector-memory-related genes. *Proc Natl Acad Sci USA* 110(26):10735–10740.
- Roychoudhuri R, et al. (2013) BACH2 represses effector programs to stabilize T(reg)-mediated immune homeostasis. *Nature* 498(7455):506–510.
- Yamada T, Park CS, Mamonkin M, Lacorazza HD (2009) Transcription factor ELF4 controls the proliferation and homing of CD8⁺ T cells via the Krüppel-like factors KLF4 and KLF2. *Nat Immunol* 10(6):618–626.