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mTORC2 regulates CD8+ effector and memory T cell differentiation through serum and glucocorticoid kinase 1 (SGK1)

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The mechanistic target of rapamycin (mTOR) is an essential regulator of T cell metabolism and differentiation. In this study, we demonstrate serum- and glucocorticoid-regulated kinase 1 (SGK1), a downstream node of mTORC2 signaling, represses memory CD8+ T cell differentiation. During acute infections, murine SGK1 deficient CD8+ T cells adopt an early memory precursor phenotype leading to more long-lived memory T cells. Thus, SGK1 deficient CD8+ T cells demonstrate an enhanced recall capacity in response to reinfection and can readily reject tumors. Mechanistically, activation of T-SGK1−/− CD8+ T cells results in decreased Foxo1 phosphorylation and increased nuclear translocation of Foxo1 to promote early memory development. Overall, SGK1 might prove to be a powerful target for enhancing the efficacy of vaccines and tumor immunotherapy.

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Author Contributions

C.H.P, E.B.H, and J.D.P. designed and oversaw the study; C.H.P, E.B.H W.X., I.H.S., M.H.O., I.M.S., R.L.B. and A.J.T. performed experiments and data analysis. J.W. helped with mouse genotyping and colony maintenance. C.H.P, E.B.H and J.D.P. wrote the manuscript.

Introduction

CD8$^+$ T cells are a major component of the adaptive immune response in response to acute infection. During the peak immune response, two distinct subsets of CD8$^+$ T cells are observed based on surface expression of the IL-7 receptor (IL-7R/CD127) and killer cell lectin-like receptor subfamily G, member 1 (KLRG1)(1–3). Short-lived terminal effector CD8$^+$ T cells (SLECs) are defined as CD127$^-$ and KLRG1$^+$, whereas memory precursor CD8$^+$ T cells are defined as CD127$^+$ and KLRG1$^-$ (MPECs). A significant amount of CD8$^+$ T cells found within the early memory precursor population will generate a stable, long-lived pool of memory T cells, poised to respond upon antigen rechallenge. Several studies over the years have defined critical signaling pathways and transcriptional mediators that influence the decision-making process of how naïve CD8$^+$ T cells differentiate into effector or memory T cells (4). mTOR is an evolutionarily conserved signaling pathway that integrates environmental cues to regulate cellular metabolism, protein synthesis, differentiation, survival, and growth (5, 6). Numerous studies have now demonstrated that both mTORC1 and mTORC2 regulate effector and memory CD8$^+$ T cell differentiation (7–9). Two studies including ours revealed an important role for mTORC2 signaling as mTORC2 deficient (Rictor knockout, T-Rictor$^{-/-}$) CD8$^+$ T cells generated more long-lived memory CD8$^+$ T cells (8, 9).

Because of the critical role of mTORC2 in regulating CD8$^+$ T cell responses, we hypothesized that a direct downstream target of mTORC2—SGK1—might also be an important regulator of effector and memory CD8$^+$ T cell differentiation as our group has previously shown that SGK1 is necessary for T_H2 CD4$^+$ T cell differentiation(10). We hypothesized that SGK1 might be responsible for the enhanced memory phenotype that we observed in T-Rictor$^{-/-}$ mice (10, 11). To this end, we utilized our SGK1 deficient (T-SGK1$^{-/-}$) mice to study the role of SGK1 in CD8$^+$ effector and memory T cell differentiation.

Materials and Methods

Mice

Mice were kept in accordance with guidelines of the Johns Hopkins University Institutional Animal Care and Use Committee. T cell conditional knockout *Sgk1* and *Rictor* mice were previously described (10). C57BL/6J, *CD4*-Cre, and CD8$^+$ OT-I transgenic mice were obtained from Jackson Laboratories and bred to CD90.1 backgrounds.

T cell stimulation and immunoblotting

Murine CD8$^+$ T cells were purified by negative selection using a CD8$^+$ isolation kit and MACS Cell Separation (Miltenyi Biotec/Biolegend). Immunoblotting for mTOR signaling was performed as previously described (10). OT-I CD8+ splenocytes were stimulated with 100ng/mL OVA I (Anaspec) for 48 hours then expanded and rested in either IL-2 or IL-7/15 (10ng/mL, Peprotech). Viable CD8$^+$ T cells were enriched through ficoll gradient for functional analysis. Human PBMCs were obtained from healthy control leukopacks.

Acute infection and tumor models

For acute infection experiments, 5e3 to 1e6 naïve CD8+ OT-1 T cells congenically marked with Thy1.1 were transferred intravenously to Thy1.2 C57/BL6 recipients by retroorbital injection. Recipients were challenged with Vaccinia-OVA or Listeria-OVA, or X31 influenza (Charles River). Mice were subcutaneously implanted with 1×10^6 EL4-OVA (ATCC). 11 days after tumor inoculation, mice received 7.5×10^5 Day 4 IL-2 activated WT or T-SGK1$^{-/-}$ OT-I CD8$^+$ T cells. Mice were randomized into groups on day of therapy. Tumor end point and volume were calculated as previously described (8).

Chromatin immunoprecipitation (ChIP)

ChIP assay was done using the MAGnify chromatin immunoprecipitation kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 10e6 Thy1.1+ adoptively transferred and sorted OT-I CD8$^+$ T cells. anti-mouse Foxo1 antibody (Cell Signaling, 2880) or rabbit IgG bound to magnetic beads. Immunoprecipitates and input fraction were analyzed by qPCR using SYBR green PCR Master Mix (Applied Biosystems), and the following primers:

IL7r forward: 5′- ACCTCATCAGCCTTTCATGG-3′,

IL7r reverse: 5'-ATCCCCTGAGCAAACTAGCA-3'

Eomes forward 5'- CAAAGAGGGCTCGTTGAGAG-3'

Eomes reverse 5'- CCTAATTCGCGTGCTTCTTT-3'

Flow Cytometric Analysis

Flow cytometry was previously described using a BD FACS Calibur or Celesta, and analyzed using FlowJo7.6 (8). Gates were determined by using unstimulated controls or isotype controls where appropriate. Cells were sorted using a BD FACS Aria.

Real-time qPCR

RNA was isolated and reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. Primer and probe sets used to detect *Cd127*, *Eomes*, and 18S rRNA were from Applied Biosystems with Taqman Universal Master Mix II using a Step One Plus Real Time PCR System.

Statistical analysis

Prism version 7.0 (GraphPad Software) was used to perform statistical analyses, including unpaired Student's t-test and 2-way Analysis of Variance (ANOVA). A p value <0.05 was statistically significant.

Results

Recognition of antigen results in activation of SGK1 in CD8$^+$ T cells

To analyze SGK1 activity upon CD8$^+$ T cell activation, we followed the kinetics of SGK1 activity by measuring the phosphorylation of its downstream target N-myc downstream-regulated gene 1 (NDRG1) (12). WT CD8$^+$ T cells activated with TCR (Signal 1) and co-stim (Signal 2) rapidly increased SGK1 activity as observed by an increase in phosphorylation of p-NDRG1 (T346) over time which closely resembles the kinetics of other mTORC1/2 downstream targets (Supplemental Fig. 1A). Likewise, T cell activation of mTORC2 deficient (T-Rictor$^{-/-}$) knockout CD8$^+$ T cells led to a significant defect in SGK1 signaling further confirming SGK1 is indeed a downstream target of mTORC2 signaling (Supplemental Fig. 1B). Loss of SGK1 activity in T-SGK1$^{-/-}$ CD8$^+$ T cells did not abolish mTORC1 signaling but unlike T-Rictor$^{-/-}$ CD8+ T cells, T-SGK1$^{-/-}$ CD8+ T cells still retained mTORC2 activity as measured by p-Akt (S473). In addition, we confirmed that SGK1 was active and downstream of mTORC2 *in vivo* by observing phosphorylation of NDRG1 in sorted effector WT CD8$^+$ T cells but not in both T-Rictor$^{-/-}$ and T-SGK1$^{-/-}$ CD8$^+$ T cells (Supplemental Fig. 1C). Therefore, we have established SGK1 as a downstream target of mTORC2 signaling upon activation of CD8$^+$ T cells *in vitro* and *in vivo*.

SGK1 inhibits early memory CD8$^+$ T cell formation during acute immune responses

Next, we wanted to determine whether mTORC2 activity through SGK1 is important in regulating CD8$^+$ T cell differentiation during an acute *in vivo* immune response. To determine whether cell-intrinsic SGK1 expression regulates the CD8$^+$ T cell response, wild type (WT) and T-SGK1$^{-/-}$ transgenic CD8$^+$ T cells specific for the class I ovalbumin (OVA) peptide (OT-I) with distinct congenic markers were co-transferred into naïve WT recipients and subsequently infected with Listeria *monocytogenes* expressing OVA (LM-OVA). On Day 7, we analyzed donor CD8$^+$ T cells in the spleen and peripheral lymph nodes, and we observed no significant differences in numbers between WT and T-SGK1$^{-/-}$ CD8$^+$ T cells (Fig. 1A). Phenotypically, T-SGK1$^{-/-}$ CD8$^+$ T cells adopted a less short-lived effector phenotype (CD127$^-$KLRG1$^+$) but an earlier memory precursor phenotype (CD127$^+$KLRG1$^-$ and CD122$^+$) compared to WT CD8$^+$ T cells (Fig. 1B, C). T-SGK1$^{-/-}$ CD8$^+$ T cells also adopted a central memory like phenotype as they expressed higher amounts of CD62L (Fig. 1D). Cytokine analysis upon peptide rechallenge revealed T-SGK1$^{-/-}$ CD8$^+$ T cells to be both potent and polyfunctional, as they produced higher amounts of IFN-γ, TNF, IL-2, and perforin compared to WT CD8$^+$ T cells (Fig. 1E). Similarly, we used an SGK1 inhibitor, EMD638683, to determine if pharmacologic targeting of SGK1 could enhance CD8$^+$ T cell differentiation (13). SGK1 inhibition in both murine and human CD8$^+$ T cells enhanced memory generation and cytokine polyfunctionality upon restimulation (Supplemental Fig. 2). These data demonstrate downstream mTORC2 signaling through SGK1 inhibits the generation of memory CD8$^+$ T cells.

Loss of SGK1 in CD8$^+$ T cells results in a superior memory pool and recall ability

Since we observed an early memory precursor phenotype in the T-SGK1$^{-/-}$ mice, we hypothesized that loss of SGK1 would ultimately lead to enhanced memory CD8$^+$ T cell

formation upon contraction. We infected WT and T-SGK1$^{-/-}$ mice with various acute infectious pathogens to analyze the formation of memory CD8^{+} T cells by monitoring antigen specific CD8^{+} T cells through tetramer analysis. We first harvested Vaccinia-OVA immunized mice to analyze the OVA specific CD8^{+} T cells in the memory pool. In support of the early display of memory precursor CD8^{+} T cells, T-SGK1$^{-/-}$ mice contained more antigen OVA specific CD8^{+} T cells in the spleen compared to WT mice (Fig. 2A). Similarly, using the LM-OVA and the X31 influenza models, T-SGK1$^{-/-}$ mice had more antigen specific CD8^{+} T cells in the memory pool demonstrating increased memory generation in various infectious models (Fig. 2B, C). A critical feature of memory cells is their prolonged survival and indeed we observed better survival of activated T-SGK1$^{-/-}$ CD8^{+} T cells compared to WT CD8^{+} T cells in the absence of any exogenous survival cytokines, which may be associated due with higher expression of the anti-apoptotic factors Bcl2 and Bcl-xL (Supplemental Fig. 3).

Finally, we wanted to determine the recall response of memory T-SGK1$^{-/-}$ CD8^{+} T cells, so we re-challenged Vaccinia-OVA immunized WT and T-SGK1$^{-/-}$ mice with LM-OVA to illicit an OVA CD8^{+} T cell specific recall. On Day 6 post-reinfection, T-SGK1$^{-/-}$ mice had more OVA specific CD8^{+} T cells compared to WT mice thereby displaying potent recall capacity of memory CD8^{+} T cells (Fig. 2D). Since increased memory potential of donor T cells is highly desired for optimal anti-tumor efficacy, we wanted to determine how T-SGK1$^{-/-}$ CD8^{+} cells would perform in a model of adoptive T cell therapy. We implanted naïve WT mice with EL4-OVA and then transferred IL-2 generated WT or T-SGK1$^{-/-}$ effector OT-I CD8^{+} T cells into tumor bearing hosts and measured tumor growth over time. Indeed, T-SGK1$^{-/-}$ CD8^{+} cells showed superior anti-tumor control with adoptive cell therapy (Fig. 2E). To support these observations and the role of memory CD8^{+} T cells in tumor immunity, we co-transferred equal numbers of congenically distinct activated WT and T-SGK1$^{-/-}$ OT-I CD8^{+} T into tumor bearing mice to analyze the response of donor CD8^{+} T cells within the same host. On Day 4 post-T cell transfer, we observed significant enrichment of T-SGK1$^{-/-}$ OT-I CD8^{+} T cells compared to WT counterparts in the tumor draining lymph node, spleen, and within the tumor microenvironment (Fig. 2F). Thus, CD8^{+} T cells lacking SGK1 signaling demonstrate enhanced differentiation into long-lived memory CD8^{+} T cells, which can also generate elevated responses upon antigen rechallenge.

SGK1 modulates Foxo1 activity in CD8^{+} T cells

Since Foxo1 is a direct downstream target of SGK1 and previous studies showed that activation of Foxo in the absence of Rictor resulted in enhanced memory formation (8, 9), we hypothesized that loss of SGK1 resulted in enhanced Foxo activity for memory differentiation. Foxo1 transcriptional activity is mediated by various phosphorylation sites primarily by Akt but also SGK1. Thus, we analyzed a known SGK1 dependent phosphorylation site (S256) on Foxo1 that controls the translocation of Foxo1 into the cytoplasm upon activation leading to transcriptional inactivation of Foxo1. We separated nuclear and cytoplasmic compartments of CD8^{+} T cells to determine how phosphorylation and nuclear localization of Foxo1 was regulated upon TCR stimulation in previously activated WT and T-SGK1$^{-/-}$ CD8^{+} T cells. Stimulated WT CD8^{+} T cells showed increased phosphorylation of Foxo1 in the cytoplasm compared to T-SGK1$^{-/-}$ CD8^{+} cells. In contrast,

T-SGK1$^{-/-}$ CD8^{+} cells demonstrated elevated total Foxo1 protein within the nucleus since Foxo1 could not traffic into the cytoplasm (Fig. 3A). We specifically observed less phosphorylation of Foxo1 (S256) in *in vivo* effector T-SGK1$^{-/-}$ OT-I CD8^{+} cells compared to WT OT-I CD8^{+} T cells in response to Vac-OVA (Fig. 3B). Foxo1 is critical in regulating expression of critical transcription factors involved in memory CD8^{+} T cell formation and maintenance such as Eomes and TCF-1 (14, 15). Indeed, on Day 7 of LM-OVA infection, we observed T-SGK1$^{-/-}$ OT-I CD8+ T cells expressing more Foxo1^{+}Eomes^{+} and TCF-1 compared to WT OT-I CD8^{+} T cells in the spleen and lymph nodes (Fig. 3C).

Since we observed an increase in these critical transcription factors, we wanted to determine if Foxo1 itself was controlling gene expression of known targets such as CD127 and Eomes. mRNA gene expression analysis of T-SGK1$^{-/-}$ CD8^{+} T cells revealed that these targets are also upregulated at the transcriptional level, consistent with our *in vivo* findings showing elevated CD127 and Eomes protein levels (Fig. 3D). Furthermore, ChIP analysis of Foxo1 in *in vivo* activated WT and T-SGK1$^{-/-}$ CD8^{+} cells revealed enhanced binding of Foxo1 to the *Il7r* and *Eomes* promoters (Fig. 3E). These findings demonstrate that SGK1 controls CD8^{+} memory T cell differentiation by regulating Foxo1 mediated transcriptional programing.

Discussion

In conclusion, we define a previously undescribed role for the mTORC2-SGK1-Foxo1 signaling axis as an essential pathway to control the transcriptional programming for CD8^{+} T cell effector and memory differentiation. By defining SGK1 as a downstream target of mTORC2, our data allows for the specific inhibition of SGK1 rather than upstream inhibition of mTORC2 and/or AKT as a means of enhancing memory formation. In addition to enhancing immunotherapy for cancer, our data has important and timely implications for enhancing the efficacy of preventative vaccines. For example, our data supports a strategy whereby elderly or immunosuppressed patients might receive COVID-19 vaccination in the setting of an SGK1 inhibitor to boost the generation of memory T cells for more long-term immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Points

1. SGK1, downstream of mTORC2 signaling, inhibits memory CD8+ T cell formation
2. Inhibiting or deleting SGK1 promotes memory CD8+ T cells and anti-tumor immunity

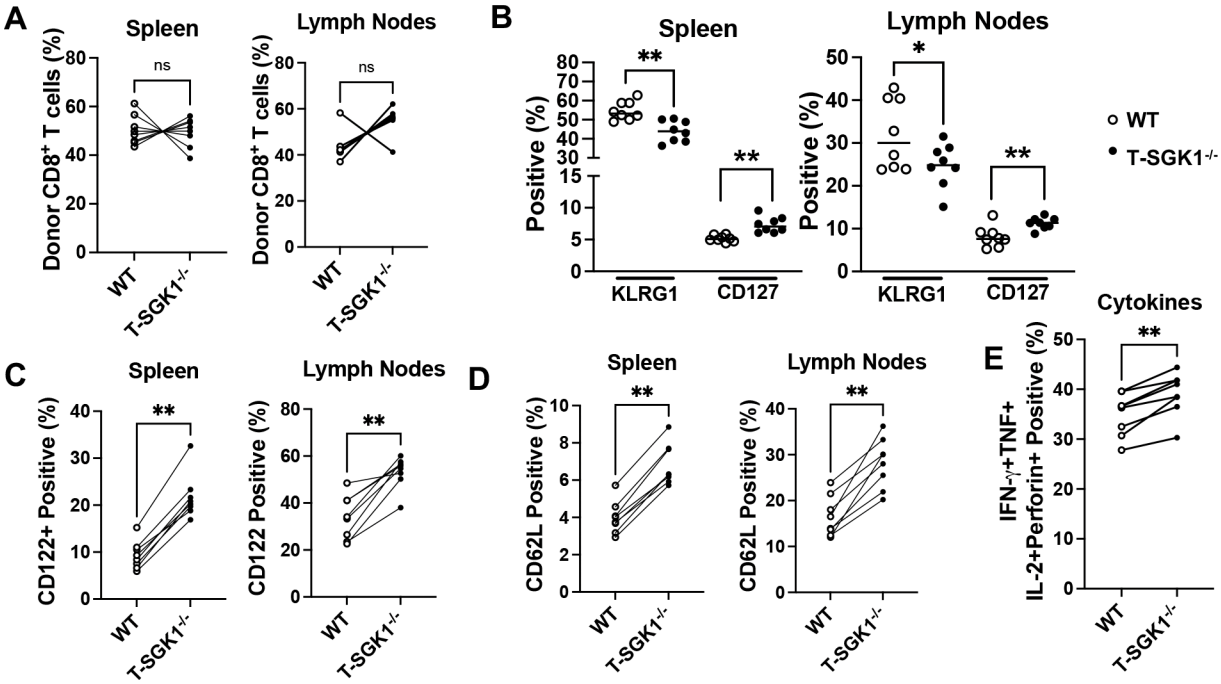

Figure 1: SGK1 deletion in CD8$^+$ T cells leads to enhanced memory precursor generation in response infection

Congenic WT (Thy1.1$^+$) and T-SGK1$^{-/-}$ (Thy1.1$^+$/1.2$^+$) OT-I CD8$^+$ T cells were co-adoptively transferred into WT Thy1.2$^+$ recipients then infected with listeria-OVA. **(A)** Percentages of donor CD8$^+$ T cells found in the spleen and peripheral lymph nodes **(B)** As in **(A)**, but percentages of single positive CD127$^+$ and KLRG1$^-$ from donor CD8$^+$ T cells **(C)** Percentage CD122 positive from donor CD8$^+$ T cells **(D)** Percentage of central memory (CD62L+) from donor CD8$^+$ T cells. All analyses were performed on viable donor Thy1.1$^+$ OT-I CD8$^+$ T cells. Experiment is representative of at least 3 independent experiments. A paired T-test (Wilcoxon T test) was performed for statistical analysis (*P <0.05, ** P < 0.01). N =8 mice

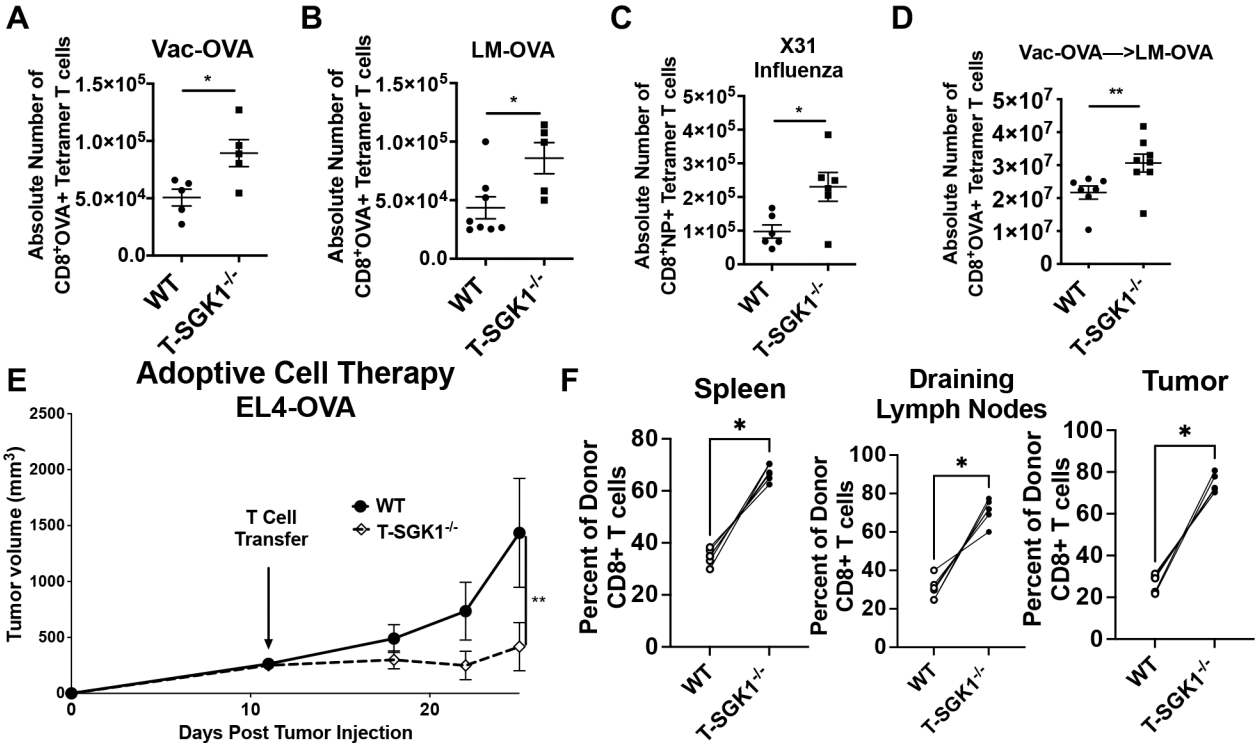

Figure 2: Absence of SGK1 in CD8$^+$ T cells promotes memory formation in response to viral and bacterial infections

(A) Statistical analysis of the absolute number of antigen specific (OVA+) CD8+ T cells in the spleen on Day 40 post Vac-OVA infection **(B)** Statistical analysis of the absolute number of antigen specific (OVA$^+$) CD8$^+$ T cells in spleen on Day 60 post LM-OVA infection. **(C)** Statistical analysis of the absolute number of antigen specific (NP$^+$) CD8$^+$ T cells in spleen on Day 80 post-infection with X31 influenza virus **(D)** WT and T-SGK1$^{-/-}$ were vaccinated with Vac-OVA for 45 days followed by re-challenge with LM-OVA for 6 days. Statistical analysis of the absolute number of antigen specific (OVA$^+$) CD8$^+$ T cells in the spleen. **(E)** WT mice were implanted with EL4-OVA then received *in vitro* generated WT and T-SGK1$^{-/-}$ OT-I CD8$^+$ cells as a form of adoptive cell therapy. Graph represents until first sacrifice **(F)** As in **(E)**, percent analysis of co-adoptive transfer of WT and T-SGK1$^{-/-}$ OT1 CD8+ in tissue from EL4-OVA bearing hosts four days post T cell transfer. Experiments are representative of at least 2 independent experiments. An unpaired t-test (Mann-Whitney) or 2-way ANOVA test (Sidak's analysis) **(E)** were performed for statistical analysis was performed for statistical analysis (*P <0.05, ** P < 0.01). N=5 or more mice per group.

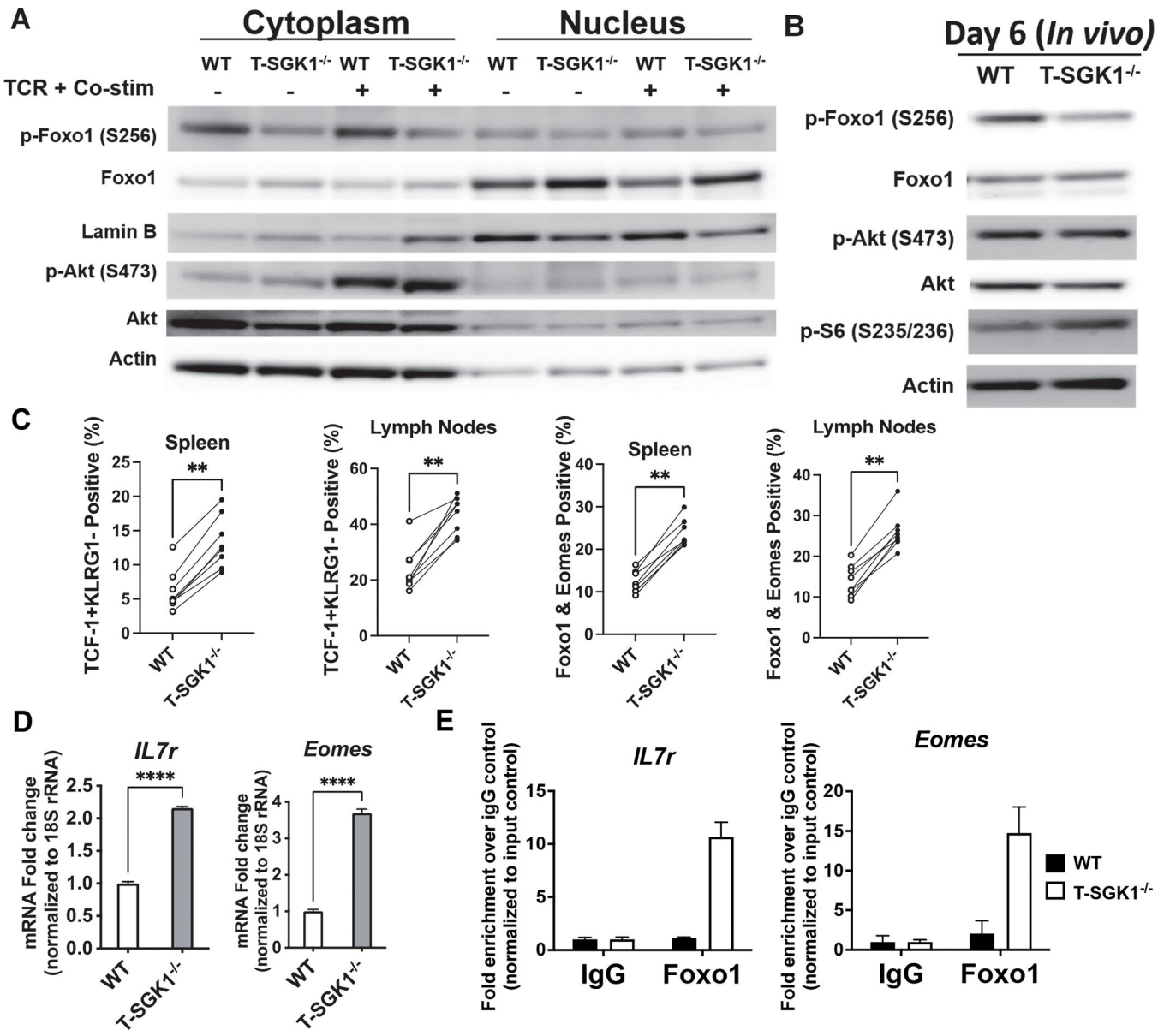

Figure 3: SGK1 mediates phosphorylation of Foxo1 to control transcriptional activity in CD8$^+$ T cells

Immunoblot analysis of cytoplasmic and nuclear fractionation of mTOR signaling in resting IL-7/15 memory WT and T-SGK1$^{-/-}$ CD8$^+$ T cells upon Signal 1 (CD3) and 2 (CD28) stimulation **(B)** As described in Fig 1C, immunoblot analysis of Foxo1 activity from sorted Day 6 *in vivo* activated WT or T-SGK1$^{-/-}$ OT-I CD8$^+$ T cells. **(C)** As in Fig 2, statistical summary of flow cytometry analysis of the percentages of TCF-1 and Foxo1 expression between WT or T-SGK1$^{-/-}$ CD8+ OTI T cells. **(D)** Statistical analysis of mRNA expression of *Il7r* and *Eomes* from sorted *in vivo* activated WT or T-SGK1$^{-/-}$ CD8+ T cells. normalized to 18s rRNA control. **(E)** Chromatin immunoprecipitation of Foxo1 at the CD127 and Eomesodermin promoters. Donor OT-I CD8$^+$ T cells were sorted as in **(D)**, and lysates were subject to ChIP analysis with Foxo1 antibody. qPCR was used to measure abundance of *IL7r*

and *Eomes* promoter sequences that were bound to Foxo1 or IgG control. Graphs depict fold enrichment of Foxo1 at the sites indicated on the *IL7r* and *Eomes* promoters, normalized to IgG control and input control. All experiments are representative of 2 independent experiments except **(C)** which is at least three times N =8 mice. A paired T-test (Wilcoxon T test) **(c)** or unpaired t-test (Mann-Whitney) was performed for statistical analysis (*P <0.05, ** P < 0.01).