The tumor suppressor menin prevents effector CD8 T-cell dysfunction by targeting mTORC1-dependent metabolic activation

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While menin plays an important role in preventing T-cell dysfunction, such as senescence and exhaustion, the regulatory mechanisms remain unclear. We found that menin prevents the induction of dysfunction in activated CD8 T cells by restricting the cellular metabolism. mTOR complex 1 (mTORC1) signaling, glycolysis, and glutaminolysis are augmented by menin deficiency. Rapamycin treatment prevents CD8 T-cell dysfunction in menin-deficient CD8 T cells. Limited glutamine availability also prevents CD8 T-cell dysfunction induced by menin deficiency, and its inhibitory effect is antagonized by α-ketoglutarate (α-KG), an intermediate metabolite of glutaminolysis. α-KG-dependent histone H3K27 demethylation seems to be involved in the dysfunction in menin-deficient CD8 T cells. We also found that α-KG activates mTORC1-dependent central carbon metabolism. These findings suggest that menin maintains the T-cell functions by limiting mTORC1 activity and subsequent cellular metabolism.
After antigen recognition, naïve T cells initiate a cell intrinsic program that induces cellular expansion and differentiation into effector T cells. The activation and differentiation of T cells is regulated in a context-dependent manner. Environmental changes cause substantial alterations in the T-cell activation and differentiation process including dysfunction. Various states of T-cell dysfunction have been reported as a consequence of altered activation and differentiation processes, being characterized by terms such as anergy, tolerance, exhaustion, and senescence.

Aging-associated dysfunction in the immune system particularly affects the T-cell compartment and is involved in the age-related decline in the immune functions, which increase the susceptibility of elderly individuals to infectious diseases and certain cancers. More recent reports on chronic viral, chemotherapy, or tumor-induced T-cell senescence have suggested that T-cell senescence was also induced in an age-independent manner. A major characteristic feature of T-cell senescence is the acquisition of a senescence-associated secretory phenotype (SASP), which is characterized by a striking increase in the secretion of pro-inflammatory cytokines, chemokines, matrix remodeling factors, and pro-angiogenic factors. These factors deleteriously alter tissue homeostasis, leading to chronic inflammation and cancer. Senescent T cells induce an increased susceptibility to autoimmune diseases such as rheumatoid arthritis through SASP. Another major alteration in senescent T cells is the impaired IL-2 production and memory formation against infection. Therefore, the efficacy of vaccination is reduced due to senescence.

The serine threonine kinase mechanistic target or rapamycin (mTOR) is a key regulator of cellular metabolism. mTOR signaling is required to integrate immune signals and metabolic cues for proper maintenance and activation of T cells. mTOR exists in two multi-protein complexes: mTORC1 and mTORC2. Both mTORC1 and mTORC2 are activated within minutes of TCR stimulation. Recent studies have revealed that mTORC1 is a major regulator of aging and cellular senescence. Rapamycin and other rapalogs specifically suppress the activity of the mammalian target of mTORC1 and decelerate cellular senescence. Furthermore, mTORC1 inhibition is considered a viable strategy for preventing T-cell dysfunction and subsequent increases in the risk of age-related diseases and it was recently reported that mTOR inhibition improves the immune function in the elderly.

Emerging evidence suggests that T cells dramatically alter their metabolic activity during T-cell receptor (TCR)-mediated activation. This change in the metabolic status is termed “metabolic reprogramming” and plays an important role in the regulation of T-cell-mediated immune responses. Similar to other non-proliferating cells, naïve T cells use fatty acid oxidation and/or a low rate of glycolysis and subsequently oxidize glucose-derived pyruvate via oxidative phosphorylation (OXPHOS) to generate ATP. Upon activation, T cells immediately shift their metabolic program to anabolic growth and biomass accumulation, which support the rapid expansion of these cells and the acquisition of the effector function, known as the Warburg effect. Although aerobic glycolysis is the dominant pathway of glucose metabolism in effector T cells, OXPHOS continues to occur. Dysregulated T-cell metabolism is associated with impaired immunity both in cancer and chronic infection.

The nonessential amino acid glutamine is the most abundant amino acid in the blood and serves as a source of carbon and nitrogen for the synthesis of proteins, lipids, and amino acids. Proliferating cells import extracellular glutamine and catabolize it via glutaminolysis in both the cytosol and mitochondria. Glutaminolysis consists of two steps. In the first step, glutamine is converted into glutamate, and in the second step, glutamate is catalyzed into the tricarboxylic acid (TCA) cycle intermediate α-ketoglutarate (α-KG), which is consumed through OXPHOS or a reductive TCA cycle. Activating and effector T cells also rapidly take up glutamine, and glutamine is required for maximizing the cell growth and proliferation of T cells. In addition, α-KG regulates the enzymatic activity of Tet methylcytosine dioxygenase 2 (TET2), Jumonji (JmjC) family histone demethylases, and PDH hydroxylases, suggesting that glutaminolysis is involved in the cellular differentiation processes.

Menin, a tumor suppressor, acts as a multifunctional scaffold protein and controls cell signaling and gene expression. Certain germinal mutations of MEN1, which encodes MENIN, cause multiple endocrine neoplasia type 1, which is an autosomal dominant syndrome characterized by concurrent parathyroid adenomas, gastroenteropancreatic tumors, and several other tumor types. Menin interacts with H3K4 methyltransferases, including mixed-lineage leukemia 1 (MLL), and is an oncogenic cofactor for MLL-associated leukemogenesis. Menin is also known to be associated with the JunD proto-oncogene product (JUND), nuclear factor of kappa light poly peptide gene enhancer in B-cells 1 (NF-kB), peroxisome proliferator-activated receptor gamma (PPAR-γ), SMAD family member 3 (SMAD3) and β-catenin, indicating its involvement in transcriptional activation and repression. It was also reported that menin localizes in the cell membrane compartment and inhibits Akt activation.

In the present study, we examined the molecular mechanism by which menin inhibits the induction of CD8 T-cell dysfunction, including senescence and exhaustion with a focus on cellular metabolism. We conclude that menin maintains the T-cell functions by limiting mTORC1 activity and subsequent cellular metabolism.

**Results**

**Dysfunction in menin-deficient activated CD8 T cells.** We previously reported that menin knockout (KO; menin<sup>flox/flox</sup> mice with CD4-Cre transgenic) naïve CD4 T cells more rapidly senesced after receiving TCR stimulation than did the wild-type (WT) control cells. Similar to menin KO naïve CD4 T cells, the growing rate of menin KO naïve CD8 T cells was reduced after day 7, even in the presence of exogenous IL-2 (Supplementary Fig. 1a). To assess the effects of menin deficiency on the cell cycle, we measured the percentage of replicating cells after incubation with 5-ethynyl-2’-deoxyuridine (EdU). A reduced number of EdU-positive cells was also detected in the menin KO CD8 T-cell cultures on days 7 (Supplementary Fig. 1b). The proportion of cell death was not increased in the menin KO CD8 T-cell cultures (Annexin V positive: approximately 14.1%) compared with that in WT cultures (Annexin V positive: 13.4%) (Supplementary Fig. 1c). The numbers of CD27<sup>low</sup>/CD62L<sup>low</sup> and CD27<sup>high</sup>/CD62L<sup>low</sup> cells were markedly increased in the menin KO CD8 T-cell cultures compared with those observed in the WT cell cultures (Fig. 1a and Supplementary Fig. 1d). Furthermore, the increased expression of inhibitory receptors, such as PD-1 was detected in the menin KO CD8 T-cell cultures (Fig. 1b and Supplementary Fig. 1e). In sharp contrast, the expression of CD226, an activating receptor, was reduced in menin KO CD8 T cells (Supplementary Fig. 1e). Moreover, the SASP-like feature was also induced in menin-deficient activated CD8 T cells. The number of OPN-producing cells was markedly increased in the menin KO CD8 T-cell cultures, whereas the generation of IFN-γ-producing cells remained unaffected (Fig. 1c and Supplementary
Menin deficiency induces dysfunction of CD8 T cells. A striking increase in IL-6, IL-10, and OPN production in the menin KO CD8 cells was detected using enzyme-linked immunosorbent assays (Fig. 1d). The augmented expression of the pro-inflammatory chemokines (Ccl2 and Ccl5), pro-inflammatory enzymes (Alox5, Ctsg, Mcpt8, and Mmp13) and pro-angiogenic factors (Pdgfa and Vegfc) was detected in menin KO effector CD8 T cells (Fig. 1e and Supplementary Fig. 1g). The strong expression of the SA β-Gal activity was detected in the menin KO effector CD8 T cells on day 12 (Fig. 1f and Supplementary Fig. 1h). The dysfunction was detected at least 3 days after the initial TCR stimulation in menin KO CD8 T cells, whereas this phenotype was not observed in WT CD8 T cells even by on day 12 (Supplementary Fig. 2). We found that these features were not detected in menin KO naive CD8 T cells (Supplementary Fig. 2). However, dysfunction was detected in menin KO CD8 T cells under stimulation with low-dose anti-TCR-β/anti-CD28 mAb (Supplementary Fig. 3). Furthermore, a similar phenotype was detected in vivo in menin KO CD8 T cells on day 7 after infection with OVA-peptide expressing Listeria monocytogenes (Lm-OVA). A decreased CD62L and CD27 expression (Fig. 1g and Supplementary Fig. 4a) and increased PD-1 (Fig. 1h and Supplementary Fig. 4b) level were detected in CD8 T cells from Lm-OVA-infected menin-deficient mice. The expression of OPN was increased in CD8 T cells from Lm-OVA-infected menin-deficient mice compared with WT mice, whereas the IFN-γ expression was comparable between the two mouse strains (Fig. 1i and Supplementary Fig. 4c). These results indicate that menin KO CD8 T cells rapidly malfunction after receiving TCR stimulation.

Menin KO CD8 T cells rapidly acquire effector functions. It was previously reported that menin localizes in the membrane compartment and inhibits Akt activation. The level of menin protein in the cytosolic fraction of aged activated CD8 T cells was lower than that in young cells, whereas the level in the nuclei was comparable (Supplementary Fig. 5). We assessed the effect of menin deficiency on the Akt signaling in activated CD8 T cells. The amount of phosphorylated (Ser473 and Thr308) was increased in menin KO activated CD8 T cells compared with WT CD8 T cells (Fig. 2a). The phosphorylation of Akt substrates was also increased in menin KO activated CD8 T cells (Supplementary Fig. 6a). Furthermore, the phosphorylation of mechanistic target of rapamycin (mTOR) (Ser2448 and Ser2481) (Fig. 2b) and ribosomal protein S6 (Ser235/236 and Ser240/244) (Fig. 2c) was enhanced in menin KO activated CD8 T cells. The
enhanced phosphorylation of S6 protein in menin KO CD8 T cells was also detected in vivo 48 h after Lm-OVA-infection (Supplementary Fig. 6b). As indicated, the Akt-mTOR complex signaling plays an important role in regulating the proliferative response and effector functions of T cells. Menin KO naive CD8 T cells adequately responded to suboptimal-dose TCR stimulation and divided more quickly than control WT cells after TCR stimulation (Fig. 2d). Furthermore, menin KO activated CD8 T cells rapidly acquired the ability to produce IFN-γ and IL-2 production (Fig. 2e). The expression of granzyme B in menin-deficient activated CD8 T cells was higher than in WT CD8 T cells (Fig. 2f). These results indicate the augmented activity of the Akt-mTOR complex 1 (mTORC1) signaling in menin KO activated CD8 T cells.

**Rapamycin prevents dysfunction in menin KO effector CD8 T cells.** We assessed whether or not the increased mTORC1 activity in menin KO activated CD8 T cells is involved in dysfunction. Rapamycin, initially identified as an antifungal metabolite produced by Streptomyces hygroscopicus, specifically suppresses the activity of mTORC1. Rapamycin was added during TCR stimulation, and then cells were expanded with IL-2 without rapamycin for the indicated number of days. The decreased expression of CD62L and CD27 in menin KO CD8 T cells was restored by rapamycin (Fig. 3a and Supplementary Fig. 7a). The expression of CD62L in WT activated CD8 T cells was also enhanced by rapamycin treatment. In addition, rapamycin restored the dysregulated cell surface expression of PD-1 and CD226 in menin KO CD8 T cells (Fig. 3b). The increased production of IL-6, IL-10, and OPN (Fig. 3c) and the generation of OPN-producing cells (Supplementary Fig. 7b) in menin KO effector CD8 T cells was partially restored by rapamycin treatment. The increased mRNA expression of Alox5, Ctg, and Mpt8 (Fig. 3d) and increased SAβ-Gal activity (Fig. 3e and Supplementary Fig. 7c) in menin KO CD8 T cells were normalized by rapamycin.

The decreased formation of immunological memory is a hallmark of immunosenescence. We previously reported the reduction in the number of memory precursor effector (CD127highKLRG1low) menin-deficient CD8 T cells during primary Lm-OVA infection and a reduction in the generation of memory CD8 T cells against OVA-peptide in menin KO mice. Therefore, we assessed the secondary immune response of menin-deficient CD8 T cells against Lm-OVA infection by adoptive transfer experiments in vivo (Supplementary Fig. 8a). In vitro-activated effector CD8 T cells with OTI transgenic (Tg) (Thy1.1+), menin KO OTI Tg (Thy1.2+) or menin KO OTI Tg (Thy1.2+) treated with rapamycin background were mixed at a 1:1 ratio and adoptively transferred into naive C57/BL6 mice (Thy1.1+ x Thy1.2+). Twenty days after adoptive transfer, the mice were infected with Lm-OVA, and the number of OT1 Tg CD8 T cells was measured. The impaired secondary immune response against Lm-OVA in menin KO CD8 T cells was restored by rapamycin treatment during primary TCR stimulation (Fig. 3f and Supplementary Fig. 8b, c). Improvement of the secondary immune response of menin KO CD8 T cells by rapamycin was confirmed by adoptive transfer experiments of memory CD8 T cells (Supplementary Fig. 9a). The impaired secondary immune response against Lm-OVA in menin KO CD8 T cells was also restored by rapamycin treatment during primary TCR stimulation under these conditions (Fig. 3g and Supplementary Fig. 9b,
Fig. 3 Rapamycin inhibits dysfunction of menin KO CD8 T cells. a) A representative staining profile of CD62L/CD27 on the cell surface of the WT and menin KO effector CD8 T cells on day 7. The percentages of cells are indicated in each quadrant. Naive CD8 T cells were stimulated with anti-TCR-β mAb plus anti-CD28 mAb with IL-2 in the presence of rapamycin for 2 days, and then the cells were further expanded with IL-2 in the absence of rapamycin for an additional 5 days. b) Representative staining profiles of CD226 and PD-1 on the surface of the cells in a. c) The ELISA for IL-6, IL-10, and OPN in the supernatants of the cells in a restimulated with immobilized anti-TCR-β for 16 h are shown with the standard deviation (n = 3: biological replicates). d) The results of the quantitative RT-PCR analysis of the pro-inflammatory enzymes in the cells in a. The results are presented relative to the expression of C32emRNA with the standard deviations (n = 3: technical replicates). e) The percentages of SA β-galactosidase (SA β-Gal)-positive cells on day 12 are shown with the standard deviation (n = 3: biological replicates). f) A 1:1 mixture of WT OT-1 Tg effector CD8 T (Thy1.1+/menin KO OT-1 Tg effector CD8 T cells (Thy1.2+) or WT (Thy1.1+)/rapamycin-treated menin KO (Thy1.2+) was adoptively transferred into WT congenic (Thy1.1+/Thy1.2+) mice. Twenty days after the transfer, the mice were infected with Lm-OVA to activate the donor cells. The donor cells were collected from the spleen on day 5 after Lm-OVA infection and analyzed by FACS. The absolute number of donor cells in the spleen was indicated (mean ± SD, n = 4 per group: biological replicates). g) WT (Thy1.1+ or Thy1.2+), menin KO or rapamycin-treated menin KO OT-1 Tg memory CD8 T cells (Thy1.2+) were mixed and transferred into WT congenic mice (Thy1.1+/Thy1.2+) as in f. The mice were infected with Lm-OVA the next day and analyzed as in f. The absolute number of donor cells in the spleen is shown (mean ± SD, n = 4 per group: biological replicates). *p < 0.05, **p < 0.01 (Student’s t-test)

These results suggest that the increased activity of mTORC1 during initial TCR stimulation is involved in the induction of dysfunction in menin KO CD8 T cells.

Enhanced central carbon metabolism in menin KO CD8 T cells. Since mTOR signaling is a metabolic cue for proper maintenance and activation of T cells, we next wanted to determine whether or not menin regulates the metabolic process in activated CD8 T cells. The incorporation of the glucose analog 2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)aminol]-D-glucose) was higher in menin KO activated CD8 T cells than in WT CD8 T cells (Fig. 4a). The intracellular level of glutamate was dramatically increased by anti-TCR-β plus anti-CD28 mAb stimulation, and the level was significantly higher in menin KO activated CD8 T cells than in WT CD8 T cells (Fig. 4b). Both the incorporation of 2-NBDG (Supplementary Fig. 10a) and upregulation of the intracellular glutamate (Supplementary Fig. 10b) in activated CD8 T cells were partially inhibited by rapamycin indicating that mTORC1 signaling is involved in the TCR-mediated activation of central carbon metabolism. To detect the metabolic status in WT and menin-deficient CD8 T cells comprehensively, naive CD8 T cells were stimulated with an anti-TCR-β mAb plus anti-CD28 mAb for 36 h and subjected to metabolic profiling of 116 metabolites. The intracellular concentration of glycolytic metabolites such as glucose 6-phosphate and fructose 1,6-diphosphate was increased in menin KO CD8 T cells (Fig. 4c). The level of lactate, an end-product of anaerobic glycolysis, also increased in menin KO CD8 T cells, while the concentration of pyruvate moderately decreased (Fig. 4c). The intracellular levels of glutamine and glutamate were significantly reduced in menin KO CD8 T cells compared to WT CD8 T cells 36 h after the initial stimulation (Fig. 4d), while the concentrations of metabolic intermediates of the TCA cycle such as succinate, fumarate and malate, increased (Fig. 4e). The levels of citrate, cis-aconitate, and isocitrate were marginally decreased (Fig. 4e), implying accelerated OXPHOS via glutaminolysis in menin KO CD8 T cells. The increased anaerobic glycolysis (extracellular acidification rates [ECAR]) (Fig. 4f) and oxygen consumption rates (OCR) (Fig. 4g) in menin KO activated CD8 T cells was confirmed using a Seahorse Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) under both...
Glutamine-deprived conditions (culture medium with low L-glutamine level in this culture medium is reduced (control: 3 mM, glutamine-deprived: 0.05 mM) but not completely depleted, as a substantial amount of L-glutamine is present in fetal calf serum. To directly confirm the role of glutamine and subsequent glutaminolysis on the induction of dysfunction, we stimulated T cells with anti-TCR-β mAb plus anti-CD28 mAb in the presence of IL-2 and cultured the cells for the first three days under glutamine-deprived conditions (culture medium with low L-glutamine). The cells were then cultured under glucose-deprived and glucose-sufficient conditions. These data indicate that both anaerobic glycolysis and glutaminolysis are facilitated in CD8 T cells by menin deficiency.

Glutamine-α-KG axis regulates CD8 T-cell dysfunction. We examined whether or not an enhanced glutamine metabolism is involved in the induction of dysfunction in menin KO activated CD8 T cells. To assess the role of glutamine metabolism, we used 6-diazo-5-oxo-L-norleucine (L-Don), a glutamine analog that antagonizes glutamine, and aminoxyacetic acid (AOA), an inhibitor of transaminases. The intracellular concentration of glutamate in activated CD8 T cells decreased by L-Don or AOA in the presence of IL-2 for 24 h. 2-NBDG was then added to the cultures for 30 min, and its incorporation was determined by FACS. A representative FACS profile is shown. The intracellular level of glutamine in naive and activated CD8 T cells with WT or menin KO background are indicated (n = 3: biological replicates). The results are presented with the standard deviation. The intracellular amount of glucose 6-phosphate (G6P), fructose 1,6-diphosphate (F1,6P), pyruvate, and lactate in the WT and menin KO activated CD8 T cells are presented with the standard deviation (n = 3: biological replicates). Naive CD8 T cells were stimulated with anti-TCR-β and anti-CD28 mAbs in the presence of IL-2 for 36 h. The intracellular amount of glutamine and glutamate of the cells in c. The results are presented with the standard deviation (n = 3: biological replicates). e The intracellular amounts of TCA cycle intermediates of the cells in c. The results are presented with the standard deviation (n = 3: biological replicates). f, g Naive CD8 T cells were stimulated with anti-TCR-β and anti-CD28 mAbs in the presence of IL-2 for 36 h, and then glycolysis (f) and the OCR (g) were determined before or 20 min after glucose (10 mM) injection. b, f, g **p < 0.01 (Student’s t-test), c, d, e *p < 0.05, **p < 0.01, ***p < 0.001 (Welch’s t-test).

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Histone H3K27 demethylation is involved in CD8 T-cell dysfunction. It has been well established that α-KG acts as a cofactor of histone and DNA demethylases. We found that the levels of histone H3K27 di-methylation (me2) and H3K27 tri-methylation (me3) in activated CD8 T cells were more sensitive to the glutamine and α-KG concentration than were the levels of H3K4me3. The levels of histone H3K27me2 and H3K27me3 in activated CD8 T cells increased under glutamine-deprived conditions and reduced in the presence of α-KG, whereas the H3K4me3 level was unaffected. The expression of utx mRNA was not detected in utx-deficient activated CD8 T cells (Supplementary Fig. 13b). The decreased expression of CD62L and CD27 in menin KO CD8 T cells was partially restored by utx deficiency (Fig. 6c and Supplementary Fig. 13c). The expression of CD62L was also enhanced in utx KO activated CD8 T cells (Fig. 6c and Supplementary Fig. 13c). The deletion of the utx reduced the cell surface expression of PD-1 in menin KO effector CD8 T cells (Fig. 6d). The increased

**Fig. 5** The glutamine-α-KG axis is involved in the dysfunction in menin KO CD8 T cells. A representative staining profile of CD62L/CD27 on the cell surface of the WT and menin KO effector CD8 T cells on day 7. The percentages of cells are indicated in each quadrant. Naive CD8 T cells were activated and cultured for 3 days under normal (Ctrl), glutamine-deprived (dGln), or glutamine-deprived supplemented with DM-α-KG (dGln/α-KG) conditions for 3 days, and then the cells were further expanded with IL-2 under normal conditions for an additional 4 days. An analysis was performed on day 7 after the initial anti-TCR-β/CD28 stimulation. The expression of CD62L/CD27 was reduced in glutamine-deprived conditions. The ELISA results for IL-6, IL-10, and OPN in the supernatants of the cells in a were restimulated with immobilized anti-TCR-β for 16 h are shown with standard deviation (n = 3: biological replicates). The results are presented relative to the expression of 33β mRNA with the standard deviations (n = 3: technical replicates). The percentages of SAβ-Gal-positive cells on day 12 after the initial anti-TCR-β/CD28 mAb stimulation are shown with the standard deviation (n = 3: technical replicates). A 1:1 mixture of WT OT-1 Tg effector CD8 T (Thy1.1+/menin KO OT-1 Tg effector CD8 T cells under normal conditions (Thy1.1+) or WT (Thy1.1+)/menin KO under glutamine-deprived conditions (Thy1.2+) was adoptively transferred into WT congenic (Thy1.1+ Thy1.2+) mice. Twenty days after the transfer, the mice were infected with Lm-OVA to activate the donor cells. The donor cells were collected from the spleen on day 5 after Lm-OVA infection and analyzed by FACS. The absolute number of donor cells in the spleen is shown (mean ± SD, n = 4 per group: biological replicates). * p < 0.05, ** p < 0.01 (Student’s t-test).
Finally, we examined the impact of cell lines. The phosphorylation of mTOR (Ser2448) and α activity in de Ctsg increased mRNA expression of SASP factors, such as initial anti-TCR- mRNA with the standard deviations (DM-ribosomal S6 protein (Ser240/244) in activated CD8 T cells results of the quantitative RT-PCR analysis of the pro-inflammation of histone H3 in the WT or menin KO CD8 T cells cultured under normal conditions for 3 days. A representative staining profile of CD62L/CD27 on the cell surface of the WT, utx KO menin KO or menin/utx-double KO effector CD8 T cells on day 7. The percentages of cells are indicated in each quadrant. Representative staining profile of PD-1 on the cell surface of the cells in reconstituted with immobilized anti-TCR-β for 16 h are shown with the standard deviation (n = 3: technical replicates). The results of the quantitative RT-PCR analysis of the pro-inflammatory enzymes of the cells in. The results are presented relative to the expression of Cd3e mRNA with the standard deviations (n = 3: technical replicates). The percentages of SA β-galactosidase (SA β-Gal)-positive cells on day 12 after the initial anti-TCR-β/CD28 stimulation are shown with the standard deviation (n = 3: biological replicates). *p < 0.05, **p < 0.01 (Student’s t-test)

production of IL-6, IL-10, and OPN in menin KO effector CD8 T cells was partially suppressed by the utx deficiency, whereas the IFN-γ production was moderately increased (Fig. 6c). The increased mRNA expression of SASP factors, such as Alox5, Ctg, and Mpt8 in menin KO CD8 T cells was normalized by the utx deficiency (Fig. 6f). In sharp contrast, the increased SA β-Gal activity in menin KO CD8 T cells was not inhibited by the Utx deficiency (Fig. 6g and Supplementary Fig. 13d). These results suggest that the α-KG-dependent demethylation of histone H3K27 is involved in the dysfunction of menin KO CD8 T cells.

α-KG activates the mTORC1 and central carbon metabolism. Finally, we examined the impact of α-KG administration on the mTORC1 activity and metabolism in effector CD8 T cells, since the α-KG-mediated activation of mTORC1 has been reported in other cell lines. The phosphorylation of mTOR (Ser2448) (Fig. 7a) and ribosomal S6 protein (Ser240/244) (Fig. 7b) in activated CD8 T cells was reduced under glutamine-deprived conditions and restored by DM-α-KG. The phosphorylated and total AMPKα level decreased in activated CD8 T cells under glutamine-deprived conditions, suggesting that the deprivation of glutamine did not induce AMPK activation in activated CD8 T cells (Supplementary Fig. 14). Furthermore, the administration of α-KG marginally enhanced the phosphorylated and total AMPKα level. These results indicate that glutamine-α-KG axis controls mTOR phosphorylation through AMPK-independent pathway. We found that DM-α-KG stimulated both ECAR (Fig. 7c) and OCR (Fig. 7d) in effector CD8 T cells cultured under glutamine-deprived conditions. The incorporation of 2-NBDG was reduced by glutamine deprivation and also restored by DM-α-KG administration (Supplementary Fig. 15a). The intracellular concentrations of glycolytic intermediates such as fructose 1,6-diphosphate (F1, 6P) and 3-phosphoglycerate (3-PG) were reduced by glutamine deprivation, and decreased levels of F1, 6P, and 3-PG under glutamine-deprived conditions were recovered by DM-α-KG addition (Supplementary Fig. 15b). The intracellular concentration of succinate, an intermediate of the TCA cycle, was also reduced by glutamine deprivation and restored by DM-α-KG (Supplementary Fig. 15b). Furthermore, the intracellular concentration of NADH and the NADH/NAD+ ratio, an indicator of the central carbon metabolism activity, were markedly reduced in the activated CD8 T cells under glutamine-deprived conditions and normalized by DM-α-KG (Supplementary Fig. 15c), suggesting the α-KG-dependent regulation of the central carbon metabolism in activated CD8 T cells. The expression of glucose transporter (Slc2a1), glycolytic enzymes (Hk2, Gapdh, and Ldha) and glutaminolytic enzymes (Got1, Psat1) was also decreased by glutamine deprivation in activated CD8 T cells, and the effect was antagonized.
by DM-α-KG (Fig. 7e). Interestingly, the α-KG-dependent induction of these enzymes was also inhibited by rapamycin (Fig. 7e). In addition, we found that α-KG-induced augmentation of glycolysis (Fig. 7f, left) and basal respiration (Fig. 7f, right) under glutamine-deprived conditions was inhibited by rapamycin.

In conclusion, menin limits mTORC1-dependent metabolic activation, and the loss of menin induces sustained activation of the central carbon metabolism, including glycolysis and glutaminolysis. α-KG production via metabolic activation and subsequent α-KG-dependent histone H3K27 demethylation seems to play a critical role in the induction of CD8 T-cell dysfunction. Furthermore, α-KG may activate mTORC1 signaling, with α-KG and mTORC1 forming a feedback loop to sustain the activation of the central carbon metabolism (Fig. 7g).

**Discussion**

In this study, we demonstrated the critical role of menin in regulating the mTORC1 signaling and cellular metabolism. The α-KG-dependent restriction of mTORC1 activity and cellular metabolism during the initial TCR-mediated activation phase seem to be important for preventing the induction of dysfunction in activated CD8 T cells. We found that the glutamine-α-KG axis plays a critical role in inducing CD8 T-cell dysfunction. The glutamine-α-KG axis and mTORC1 seem to form a positive feedback loop to sustain the central carbon metabolism. mTORC1 activity is required for the activation of glycolysis and glutaminolysis, while glutamine and α-KG are required for the sustained activation of mTORC1 in activated CD8 T cells. These results imply that the level of glutamine metabolism during the antigen recognition phase may determine the CD8 T-cell fate through modulation of the mTORC1 activity. Our present data, therefore, suggest the critical role of menin in regulating the cellular metabolism and the subsequent fate decision of activated CD8 T cell.

It was reported that 2-hydroxyglutarate (2-HG), a metabolite of α-KG, activates the mTOR-signaling pathway. 2-HG then leads to the activation of mTOR by decreasing the protein stability of the DEP domain-containing mTOR-interacting protein (DEPTOR), a negative regulator of mTORC1/2. Furthermore, the accumulation of R- and S-2-HG in CD8 T cells after receiving TCR stimulation was reported. The S-2-HG produced in antigen-stimulated CD8 T cells appears to be derived from extracellular glutamine and is produced by lactate dehydrogenase A (Ldha) and/or malate dehydrogenase. We demonstrated that the expression of Ldha was reduced by glutamine deprivation and induced in an α-KG-
dependent manner in activated CD8 T cells. We also showed that the ECAR was increased by α-KG in CD8 T cells cultured under glutamine-deprived conditions, indicating the augmented enzymatic activity of Ldha by α-KG. In addition, we found that the concentration of 2-HG was significantly higher in menin-deficient activated CD8 T cells than in WT CD8 T cells, and the 2-HG level was reduced by glutamine deprivation and restored by α-KG supplementation in WT activated CD8 T cells (Supplementary Fig. 16). We, therefore, speculated that α-KG activates the mTORC1-signaling pathway in CD8 T cells through enzymatic conversion to 2-HG.

Senescent CD8 T cells are found within the CD27−CD28− population, and these terminally differentiated T cells can be subdivided into two populations: CD45RA+ or CD45RA−. CD27−CD28−CD45RA+ T cells have multiple features of senescence, including a low proliferative activity and SASP. In addition, one of the most prominent changes in T cells in elderly people is the progressive accumulation of highly differentiated effector memory T (T EM) cells (CD45RA−CD28−). It was reported that human immunodeficiency virus-1 and human cytomegalovirus chronic infection induces CD27−CD28−CD45RA+ senescent CD8 T cells in an age-independent manner. These findings suggest that chronic and/or robust stimulation with antigen and/or cytokine accelerates T cell senescence in an age-independent manner. We demonstrated that CD62L and CD27 expression was rapidly reduced in menin KO CD8 T cells after receiving antigenic stimulation in vivo and in vitro. We also confirmed that some portion of CD62L+CD27low menin KO CD8 T cells had a down-regulated CD28 expression (Supplementary Fig. 17). Furthermore, the cytosolic menin protein was decreased in aged effector CD8 T cells. Thus, menin may inhibit T-cell senescence, and menin-deficient mice may be useful as an antigen-induced as well as aging-induced T-cell senescence model.

α-KG regulates the enzymatic activity of TET2, JmJc family histone demethylases and PDH hydroxylases. We found that α-KG preferentially reduced histone H3K27me2/3 methylation in activated CD8 T cells. The methylation level of histone H3K27me2/3 was decreased in menin KO activated CD8 T cells, and the deletion of the utx gene partially restored dysfunction in menin KO CD8 T cells. These results indicate that utx-dependent histone H3K27 demethylation is involved in the induction of dysfunction in menin KO CD8 T cells. It was reported that the H3K27 demethylase UTX-1, an orthologue of mammalian Utx, inactivated histone H3K27 demethylation is involved in the induction of senescence, including a low proliferative activity and SASP. We, therefore, speculate that α-KG might inhibit T-cell senescence by reducing histone H3K27me2/3 levels in CD8 T cells cultured under glutamine-deprived conditions.

**Methods**

**Mice.** Menin−/−, Cre TG mice and mice under the control of the Cd4 promoter were purchased from The Jackson Laboratory. Utx−/− mice were established by Drs. Kazuaki Inoue and Yuuki Imai (Ehime University). Gene-manipulated mice with C57BL/6 background were used in all experiments. C57BL/6 mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Female mice were used in the in vivo experiments. Both male and female mice were used in the in vitro experiments. All mice were maintained under specific-pathogen-free conditions and were used at 8–12 weeks of age. All of the animal experiments received approval from the Ehime University Administrative Panel for Animal Care. All animal care was conducted in accordance with the guidelines of Ehime University.

**Reagents and antibodies.** Rapamycin was purchased from Wako Chemicals (cat#01961; Osaka, Japan). Dimethyl alpha-ketoglutarate (DM-aKG) was obtained from Tokyo Chemical Industry (cat#K0013; Tokyo, Japan). Antibodies used for immunoblotting were as follows: anti-Phospho-S6 (Ser240/244) Alexa Fluor 647 mAb (cat#5044; Cell Signaling Technology), anti-Carbonic Anhydrase II (CA II) mAb (cat#39157; Active Motif), anti-histone H3K4me3 mAb (cat#61379; Active Motif, Carlsbad, CA, USA), anti-Phospho-S6 (Ser235/236)-Alexa Fluor 647 mAb (cat#4851; Cell Signaling Technology), anti-CD27-PE mAb (cat#558754; BD Bioscience, San Jose, CA, USA), anti-IFN-γ-fluorescein isothiocyanate (FITC) mAb (cat#554411; BD Bioscience), IL-2−APC mAb (cat#558049; R&D Systems, Minneapolis, MN, USA), anti-IFN-γ-fluorescein isothiocyanate (FITC) mAb (cat#554411; BD Bioscience), IL-2−APC mAb (cat#565654; Thermo Fisher Scientific), anti-Phospho-STAT1 (Y701)-Alexa Fluor 647 mAb (cat#4851; Cell Signaling Technology), anti-CD45RA-PE-Cy7 mAb (cat#15630-080; Thermo Fisher Scientific) and anti-Phospho-CD27 mAb (cat#19271; Cell Signaling Technology). The antibody for immunoblotting was as follows: anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), anti-α-Tubulin (cat#37008; Cell Signaling Technology), and anti-α-Tubulin (cat#37008; Cell Signaling Technology).

**Immunoblotting analyses.** The CD8 T cells were lysed directly with sodium dodecyl sulfate (SDS) sample buffer (0.1 M Tris-HCl, 20 γ Ci/L, 4% SDS, 0.004% bromophenol blue, 50 mM dithiothreitol (DTT)) and sonicated to shear.
DNA. The lysates were separated on an SDS polyacrylamide gel and then subjected to immunoblotting with specific antibodies.

**Incorporation of 2-NBDG.** The CD8 T cells were pulsed with 50 μM 2-NBDG (cat#N131395; Thermo Fisher Scientific) in non-glue media (cat#185-02864; Wako Chemicals) for 30 min at 37 °C, and the incorporation of 2-NBDG into the cells was assessed by FACS.

**Metabolic profiling.** Metabolome measurements and data processing were performed through a facility service at Human Metabolome Technology Inc. (Yamagata, Japan). Briefly, naïve CD8 T cells were stimulated with plate-bound anti-TCR mAb plus anti-CD28 mAb in the presence of IL-2 for 36 h. The cells (3 x 10^6 cells) were washed with 5% (w/v) mannitol and then lyzed with 800 μl of methanol and 500 μl of Milli-Q water containing internal standards (H3304-1002, Human Metabolome Technology Inc.) and left to rest for another 30s. The extract was obtained and centrifuged at 2300 × g for 12 min at 4 °C to remove proteins. The filtrate was centrifugally concentrated and re-suspended in 50 μl of Milli-Q water for the capillary electrophoresis-mass spectrometry (CE-MS). Cationic compounds were measured in the positive mode of capillary electrophoresis-time of flight-mass spectrometry (CE-TOFMS) and anionic compounds were measured in the positive and negative modes of capillary electrophoresis-tandem mass spectrometry (CE-MS/MS) in accordance with the methods developed by Soga et al. In some experiments, the glutamate concentration was determined using a Glutamate Colorimetric Assay kit; cat#103010-100; Agilent Technologies).

**Detection of senescence-associated β-galactosidase activity.** CD8 T cells were cultured for 12 days as described above, and then an SA-β-galactosidase assay was performed using a Senescence β-Galactosidase Staining Kit (cat#89860; Cell Signaling Technology).

**Adoptive transfer of CD8 T cells and Listeria infection.** To assess the phenotype of in vivo activated antigen-specific CD8 T cells, naïve CD8 T cells were prepared from the spleens of WT OT-I Tg (Thy1.2+) or OT-1 Tg mice (Thy1.2+) and intravenously transferred into naive C57BL/6 (Thy1.1+) mice. The CD8 T cells were pulsed with 50 μg/ml of Lm- OVA strain at 5 x 10^3 CFU. The donor cells were prepared and analyzed on day 5 after infection. In several experiments, the donor cells were isolated from the spleen after infection. In several experiments, the donor cells were isolated from the spleen after infection. In several experiments, the donor cells were isolated from the spleen after infection. In several experiments, the donor cells were isolated from the spleen after infection.

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