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Senescent CD8$^+$ T cells acquire NK cell-like innate functions to promote antitumor immunity

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Abstract

It has been suggested that aging of the immune system (immunosenescence) results in a decline in the acquired immune response, which is associated with an increase in age-related tumorigenesis. T-cell senescence plays a critical role in immunosenescence and is involved in the age-related decline of the immune function, which increases susceptibility to certain cancers. However, it has been shown that CD8$^+$ T cells with the senescent T-cell phenotype acquire an natural killer (NK) cell-like function and are involved in tumor elimination. Therefore, the role of senescent CD8$^+$ T cells in tumor immunity remains to be elucidated. In this study, we investigated the role of senescent CD8$^+$ T cells in tumor immunity. In a murine model of transferred with B16 melanoma, lung metastasis was significantly suppressed in aged mice (age ≥30 weeks) in comparison to young mice (age 6–10 weeks). We evaluated the cytotoxic activity of CD8$^+$ T cells in vitro and found that CD8$^+$ T cells from aged mice activated in vitro exhibited increased cytotoxic activity in comparison to those from young mice. We used *Menin*-deficient effector T cells as a model for senescent CD8$^+$ T cells and found that cytotoxic activity and the expression of NK receptors were upregulated in *Menin*-deficient senescent CD8$^+$ T cells. Furthermore, *Menin*-deficient CD8$^+$ T cells can eliminate tumor cells in an antigen-independent manner. These results suggest that senescent effector CD8$^+$ T cells may contribute to tumor immunity in the elderly by acquiring NK-like innate immune functions, such as antigen-independent cytotoxic activity.

KEYWORDS
antigen-independent cytotoxicity, immunosenescence, Menin, senescent CD8$^+$ T cells, T-cell innate functions

Abbreviations: B6, C57/BL6; ChIP, chromatin immunoprecipitation; OT-1, OT-1 T-cell receptor; OVA, ovalbumin; RNA-seq, RNA sequencing; TCR, T-cell receptor.

1 | INTRODUCTION

During differentiation in the thymus, T cells acquire diverse T-cell receptor (TCR) repertories through reorganization of the TCR genes and subsequent positive and negative selection. The supply of naïve T cells from the thymus is thought to be reduced in the elderly because the thymus atrophies with age[1,2] Furthermore, repeated invasion of the same antigen over a prolonged survival period reduced the diversity of the TCR repertoire.[3,4] T-cell numbers in the periphery themselves are maintained by homeostatic proliferation.[5] However, it is generally believed that the immune response against novel antigens is weakened in older individuals, due in part to a bias in the TCR repertoire, which leads to an increase in age-related carcinogenesis and chronic infections.

Age-induced immunity defects are generally viewed as detrimental and designated as "immunosenescence".[6] One of the major alterations in immunosenescence is the impaired primary CD8$^+$ T-cell response against infection and reduced vaccine efficacy due to senescence.[7] Therefore, T-cell senescence plays a critical role in immunosenescence and is involved in the age-related decline of the immune function, which increases susceptibility to infectious diseases and certain cancers.[8,9] Another alteration in immunosenescence 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.[10,11] These factors deleteriously alter tissue homeostasis, leading to the development of cancer and chronic inflammation.[10,12–14] Senescent T cells induce increased susceptibility to autoimmune diseases, such as rheumatoid arthritis, through SASP.[7,15–17] However, it has been shown that senescent CD4$^+$ T cells with cytotoxic activity are increased in supercentenarians, who live to be over 110 years of age, and that they may be involved in eliminating cancer. There is also report that CD8$^+$ T cells with the senescence phenotype acquire an natural killer (NK) function and are involved in tumor elimination.[18] Therefore, it is still unclear how senescent CD8$^+$ T cells act in antitumor responses.

We have previously reported that the tumor suppressor Menin inhibits T-cell senescence, and that *Menin*-deficient CD4$^+$ T and CD8$^+$ T cells can be used as models of senescent T cells.[19–21] Menin acts as a multifunctional scaffold protein and controls various cell signaling pathways and the expression of various genes such as *HOX* and *PTN*.[22] Germinal mutations of *MEN1*, which encodes MENIN, cause multiple endocrine neoplasia type 1,[23] which is an autosomal dominant syndrome characterized by concurrent parathyroid adenomas and gastroenteropancreatic tumors.[24] We have reported that Menin controls central carbon metabolism in CD8$^+$ T cells by the inhibitory regulation of Akt/mTOR signaling, and that Menin deficiency in activated CD8$^+$ T cells induces premature senescence, in part by enhancing central carbon metabolism. Moreover, Menin interacts with H3K4 methyltransferase complexes, including mixed-lineage leukemia 1 (MLL1).[25–27] Menin is also known to be associated with several transcription factors, including the JunD proto-oncogene product (JUND), nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB), peroxisome proliferator-activated receptor gamma (PPAR-γ), SMAD family member 3 (SMAD3), and β-catenin.[28,29]

In this study, we first demonstrated that the expression of Menin was reduced in senescent CD8$^+$ T cells generated in vitro. We then examined the role of senescent CD8$^+$ T cells in tumor immunity, mainly using *Menin*-deficient effector CD8$^+$ T cells as a model. As a result, we newly found that senescent T cells may play an important role in tumor immunity in aged individuals by acquiring NK-like innate functions (i.e., antigen-independent cytotoxic activity).

2 | MATERIALS AND METHODS

2.1 | Mice

C57BL6 mice were purchased from Clea Japan, Inc. C57BL6 mice of 6–10 weeks of age and ≥30 weeks of age were used as young mice and old mice, respectively. *Menin$^{flox/flox}$* mice and Cre transgenic (Tg) mice under the control of the *Cd4* promoter were purchased from The Jackson Laboratory. In addition, we generated WT OT-1 Tg mice or *Menin* KO OT-1 Tg mice by crossing WT mice or *Menin* KO mice with OT-1 Tg mice. WT and *Menin* KO mice of 10–16 weeks of age were used in in vivo experiments. All the animal experiments received approval from the Ehime University Administrative Panel for Animal Care.

2.2 | Reagents and antibodies

The antibodies used for cell surface and intracellular staining are described in Appendix S1.

2.3 | In vivo B16 melanoma transfer

B16 melanoma cells (2×10^5 cells in 100 μL of PBS per mouse) were intravenously injected through the tail vein. Lungs were removed and photographed front and back on day 14 after inoculation. The regions of lung metastasis appeared grossly black. The percentage of black area in the lungs was calculated using the Image J software program.

2.4 | Depletion of NK cells in vivo

NK cells were depleted by intraperitoneal administration of anti-asialo GM1 pAb (cat#146002; BioLegend). Normal rabbit serum (cat#140–06571; Wako) was used as a control. In brief, antibodies

(50 μL) were administered intraperitoneally 1 day before transplantation of B16 melanoma cells.

2.5 | Depletion of T cells in vivo

$CD8^+$ or $CD4^+$ T cells from mice with B16 lung metastasis were depleted of antibodies. $CD8^+$ T cells and $CD4^+$ T cells were depleted using anti-CD8 mAb (cat#100767; BioLegend) and anti-CD4 mAb (cat#100461; BioLegend), respectively. These antibodies were administered intraperitoneally on day -1 (200 μg), day 2 (150 μg) and day 5 (150 μg) when B16 cells were transferred. The lungs were removed on day 14 after B16 inoculation and photographed.

2.6 | $CD8^+$ T-cell stimulation and differentiation in vitro

$CD8^+$ T cells were prepared from spleen using a MojoSort Mouse $CD8^+$ T cell isolation kit (cat#480035; BioLegend). In naïve $CD8^+$ T ($CD44^{low}CD62L^{high}$) cell preparation, biotin anti-CD44 mAb (cat#103004; BioLegend) was added. In the case of total $CD8^+$ T cells, anti-CD44 mAb was not added. After isolation, the cells (7.5×10^5) were stimulated with immobilized anti-TCR-β mAb (3 μg/mL, H57-597; BioLegend) and anti-CD28 mAb (1 μg/mL, 37.5; BioLegend) with IL-2 (10 ng/mL; cat#575406; BioLegend) for 2 days. The cells were then transferred to a new plate and further cultured with IL-2 (10 ng/mL) for 5 days.

2.7 | In vitro killing assay

EL4 is a thymoma cell line in mice. E.G7 is OVA expressing EL4. $CD8^+$ T cells were effector cells (E) and tumor cells were target cells (T). E.G7 and EL4 were labeled with 0.1 μM and 1 μM Cell Proliferation Dye eFluor 670 (cat#65–0840; BioLegend), respectively. $CD8^+$ T cells (0, 0.1, 0.3, 1×10^5 cells) were co-cultured with a 1:1 mixture of E.G7 and EL4 cells (1×10^5 cells) in a 96-well U-bottom plate for 6 h. The number of surviving E.G7 and EL4 cells was analyzed by flow cytometry. The percentage of surviving cells (sCell) was calculated using the following formula: sCell (%) = sCell/average of sCell (E:T = 0:1) × 100. Cytotoxic rate (%) was calculated as follows: 100 – sCell (%).

2.8 | Library preparation and sequencing

Total RNA was extracted from $CD8^+$ T cells obtained from young and aged mice on day 7 after TCR stimulation using an RNeasy Plus Micro Kit (Qiagen). Purified RNA samples were used to prepare cDNA libraries using a QuantSeq 3′mRNA-Seq Library Prep Kit for Illumina (Lexogen). Sequencing of the samples was performed using a NextSeq500 instrument (Illimuna) using a NextSeq 500/550 High Output Kit v2.5 (Single-Reads 75 bp).

2.9 | RNA-sequencing analyses

CLC Genomics workbench 22.0 (Qiagen) was used to trim single reads and align them to the mm10 mouse reference genome. The gene expression was determined by the score of Transcripts Per Million (TPM) mapped reads. Heatmapper was used to visualize the gene expression (http://www.heatmapper.ca).[30] The data were deposited in the GSE214771 and GSE215391 databases.

2.10 | Intracellular staining

The cells were stimulated with or without an immobilized anti-TCR-β mAb (3 μg/mL) for 6 h with monensin (2 μM, cat#M5273; Sigma-Aldrich). Intracellular staining was then performed as previously described.[31] Flow cytometry was performed using a Gallios instrument (Beckman Coulter), and the results were analyzed using the FlowJo software program (BD Biosciences).

2.11 | Quantitative reverse transcripts PCR

Total RNA was isolated using TRI reagent (cat#TR118; MOR, OH, USA), and complementary DNA (cDNA) was synthesized using a superscript VILO cDNA synthesis kit (cat#11754; Thermo Fisher Scientific). Quantitative reverse transcript PCR (qRT-PCR) was performed using a Step One Plus Real-Time PCR System (Thermo Fisher Scientific). The primers are described in Appendix S2.

2.12 | Immunoblotting

The cell lysates were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (cat#78833; Thermo Fisher Scientific). The lysates were separated on an SDS polyacrylamide gel and then subjected to immunoblotting with specific antibodies. The antibodies for immunoblotting were as follows: anti-Menin antibody (cat#A300-105A; Bethyl Laboratories), anti-beta Actin antibody (cat#4970; Cell Signaling Technology).

2.13 | Library preparation and processing of ChIP-seq data

The Magna ChIP kit was used for the ChIP assay (EMD-Millipore). Anti-Menin antibody and anti-histone H3K27 ac antibody (Cat#39133; Active motif) were used. The library for ChIP-sequencing (ChIP-seq) was prepared using the NEBNext ChIP-seq Library Prep Master Mix Set for Illumina (NEB) and sequenced using Hiseq 1500 (single reads 50 bp). The data analyses were performed as previously described.[32] For visualization of the ChIP-seq results, the data were converted to a wiggle file format and were uploaded to the IGV platform (Broad Institute).

2.14 | Statistical analyses

Two-tailed unpaired *t*-tests and a one-way analysis of variance with Tukey's multiple comparison test were conducted using the Prism 9 software program. A value of $P<0.05$ was considered statistically significant.

3 | RESULTS

3.1 | CD8$^+$ T cells from aged mice exhibit antigen-independent NK cell-like cytotoxic activity

First, we addressed the differences in antitumor activity in aged (age ≥30 weeks) and young (age 6–10 weeks) mice using a B16 melanoma lung metastasis model. B16 melanoma was intravenously transferred from the tail vein into mice, and the amount of lung metastasis was measured on day 14 after transfer. As shown in Figure 1A, lung metastases were significantly reduced in aged mice in comparison to young mice. To assess the involvement of NK cells, we depleted NK cells by administering anti-asialo GM1 antibody and performed a B16 melanoma lung metastasis assay (Figure 1B). The depletion of NK cells with a high asialo GM1 expression was confirmed in the spleen (Figure S1A). Although the numbers of CD8$^+$ T cells moderately expressing asialo GM1 were increased in aged mice, asialo GM1$^+$ CD8$^+$ T cells showed only a partial reduction in number by anti-asialo-GM1 antibody treatment (Figure S1A). The lung metastases were increased in both NK cell-depleted young and aged mice; however, NK cell-depleted aged mice still had fewer lung metastatic melanomas than younger mice (Figure 1C). We performed in vitro killing assay to compare cytotoxic activity of CD8$^+$ T cells from young mice and those from aged mice using OT-1 TCR, which specifically recognize ovalbumin peptide, transgenic mice. To determine the OVA antigen-specific cytotoxic activity, an OVA-expressing EL4 thymoma (E.G7-OVA) cell line was used as a target. Antigen-specific cytotoxic activity in effector CD8$^+$ T cells from aged mice

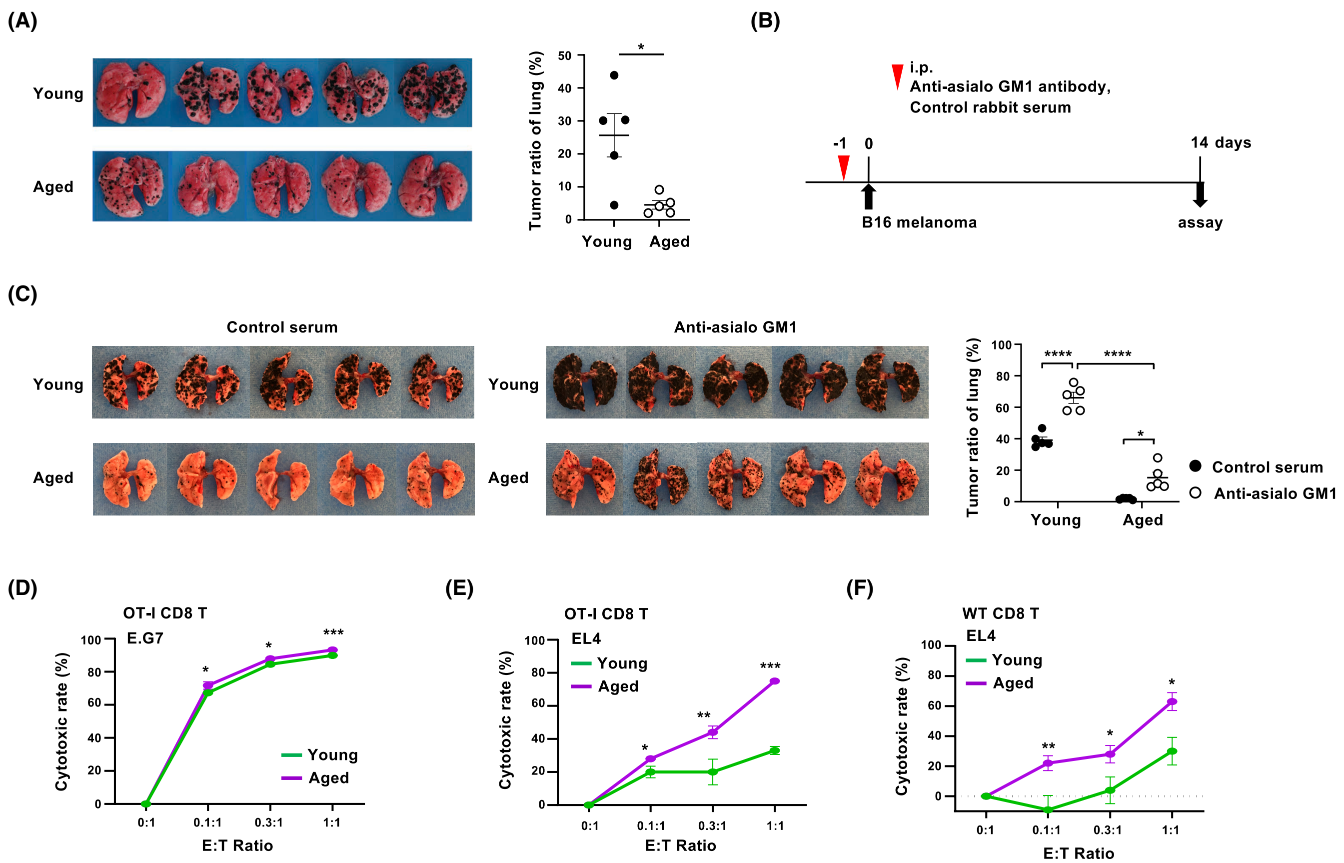

FIGURE 1 Increased antitumor activity in aged mice and CD8$^+$ T cells from aged mice. (A) The amounts of B16 lung metastasis on day 14 after tumor inoculation in young mice (6–10 weeks, $n=5$ per group) and aged (≥30 weeks, $n=5$ per group) mice. (B) Experimental design of B16 lung metastasis in mice with NK cell depletion. (C) Lung metastasis of B16 melanoma on day 14 after tumor transplantation in NK cell-depleted young and aged mice ($n=5$ per group). (D) and (E) CD8$^+$ T cells were prepared from young and aged OT-1 Tg mice. The CD8$^+$ T cells were stimulated with anti-TCR-β mAb plus anti-CD28 mAb with IL-2 for 2 days, and then the cells were further expanded with IL-2 for 5 days. CD8$^+$ T cells and tumor cells were co-cultured for 6 h. The E:T ratio was set to 0:1, 0.1:1, 0.3:1, and 1:1, respectively. The results of a killing assay of OT-1 Tg CD8$^+$ T cells against E.G7 (D) and EL4 (E). (F) The results of a killing assay of non-Tg CD8$^+$ T cells on day 7 after initial stimulation. The results of (D–F) are based on triplicate wells of a single representative experiment. The error bar displayed indicates the mean ± SEM (A, C and D–F). *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$, two-tailed unpaired *t*-test (A and D–F) and a one-way ANOVA with Tukey's multiple comparison test (C).

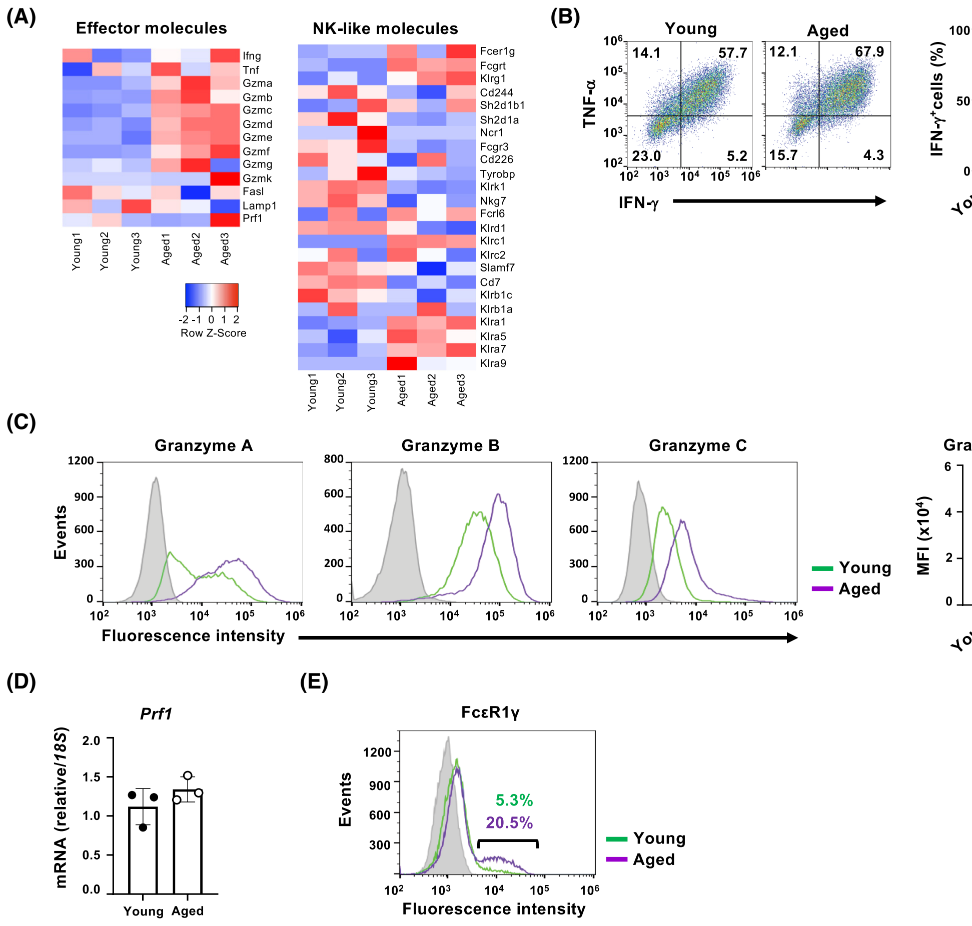

FIGURE 2 The increased expression of granzymes in CD8$^+$ T cells from aged mice. CD8$^+$ T cells were prepared from young (6–10 weeks) and aged (≥30 weeks) mice. The cells were stimulated and cultured in as Figure 1. (A) A heatmap of the expression of CD8$^+$ effector- and NK-related genes in young and aged CD8$^+$ T cells was determine by RNA-seq ($n=3$ biological replicates). (B) Representative results of intracellular staining of IFN-γ and TNF-α in the cells ($n=3$ biological replicates). (C) Representative results of intracellular staining of Gzm A, Gzm B, and Gzm C in the cells ($n=3$ biological replicates). (D) The *Prf1* mRNA expression in the cells. The gene expression levels were determined by qRT-PCR. The results are presented relative to the expression of 18 s rRNA ($n=3$ technical replicates). (E) Representative staining profile of FcεR1γ. The percentages of FcεR1γ$^+$ cells are shown. The results of (B)-(E) are representative of at least three independent experiments with similar results. The error bar displayed indicates the mean ± SD (B–D). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ (two-tailed unpaired *t*-test).

was slightly but significantly higher than that of CD8$^+$ T cells from young mice (Figure 1D). Surprisingly, OT-1 CD8$^+$ T cells from aged mice showed high cytotoxic activity against EL4 cells that did not express OVA (Figure 1E). In contrast, OT-1 CD8$^+$ T cells from young mice showed less cytotoxic activity against EL4 cells. Furthermore, CD8$^+$ T cells from non-TCR Tg aged B6 mice also showed antigen-independent cytotoxic activity against EL4 (Figure 1F).

3.2 | The increased expression of effector molecules in CD8$^+$ T cells from aged mice

We performed RNA sequencing (RNA-seq) using effector CD8$^+$ T cells generated in vitro to elucidate the molecular mechanisms underlying the increased antigen-independent cytotoxic activity in CD8$^+$ T cells from aged mice. The results of RNA-seq revealed that the mRNA expression of effector CD8$^+$ T cell- and several NK cell-related molecules was increased in CD8$^+$ T cells from aged mice in comparison to those from young CD8$^+$ T cells (Figure 2A). The intracellular staining revealed that the ratio of IFN-γ- and TNF-α- producing cells in CD8$^+$ T cells from aged mice were comparable to those from young mice (Figure 2B). The expression of cytotoxic granules, including granzyme A, granzyme B, and granzyme C, was higher in CD8$^+$ T cells from aged mice (Figure 2C). In contrast, no difference in the expression of *perforin* mRNA was observed between aged and young mice (Figure 2D). Interestingly, some effector CD8$^+$ T cells from aged mice expressed the γ subunit of the high affinity IgE receptor (FcεRI) (Figure 2E).

3.3 | Effector CD8$^+$ T cells from aged mice exhibit a senescence-like phenotype

It has been reported that senescent CD8$^+$ T cells have an enhanced inflammatory function and increased expression of NK cell-related genes.[33] We examined whether in vitro generated effector CD8$^+$ T cells from aged mice undergo cellular senescence. It is generally known that cellular senescence is accompanied by cellular hypertrophy and flattening.[34] As shown in Figure 3A, flattening and hypertrophy of cells was observed in CD8$^+$ T cells from aged mice at day 12 after in vitro anti-TCR-β mAb plus anti-CD28 mAb stimulation in the presence of IL-2. The number of senescence-associated β-galactosidase (SA-βgal) positive cells was also markedly increased in CD8$^+$ T cells derived from aged mice (Figure 3B). In senescent CD8$^+$ T cells, the expression of both CD27 and CD62L is decreased, and the expression of inhibitory receptors such as PD-1 and Tim3 is increased.[35–37] The ratio of CD27^{low} CD62L^{low} cells was higher in cultured CD8$^+$ T cells from aged mice than those from young mice (Figure 3C). In addition, the expression of PD-1 and Tim3 was also increased in cultured CD8$^+$ T cells from aged mice in comparison to those from young mice (Figure 3D). We have previously reported that *Menin*-deficient T cells express senescent T-cell-like phenotype after antigen stimulation.[19–21] We found that the protein expression of Menin in the nucleus of cultured CD8$^+$ T cells from aged mice was decreased in comparison to that in cultured CD8$^+$ T cells from young mice (Figure 3E). These results indicate that cellular senescence is rapidly induced in CD8$^+$ T cells from aged mice after TCR stimulation in vitro.

3.4 | Increased NK-like cytotoxic activity in Menin-deficient CD8$^+$ T cells

To assess whether the reduction of the Menin expression in cultured CD8$^+$ T cells from aged mice is associated with increased antitumor activity and the acquisition of NK cell-like innate immune functions, we subsequently analyzed T cell-specific *Menin*-deficient (*Menin* KO) mice. Lung metastasis of B16 melanoma was significantly reduced in *Menin* KO mice in comparison to WT mice (Figure 4A). We then performed a cell depletion assay using the administration of anti-CD8 mAb or anti-CD4 mAb to determine whether the decrease in lung metastasis of B16 melanoma in *Menin* KO mice was mediated by CD8$^+$ T cells (Figure 4B). As shown in Figure 4C, the removal of CD8$^+$ T cells by the administration of anti-CD8 mAb canceled the reduction of B16 melanoma lung metastasis in *Menin* KO mice (Figure 4C). In contrast, the elimination of CD4$^+$ T cells by anti-CD4 antibody administration failed to reverse the tendency for decreased B16 melanoma lung metastasis in *Menin* KO mice (Figure 4D). Next, we performed an in vitro killing assay to confirm the presence of increased NK cell-like cytotoxic activity in *Menin* KO effector CD8$^+$ T cells. In a cytotoxic assay targeting the EL4 thymoma cell line, *Menin* KO effector CD8$^+$ T cells showed higher cytotoxic activity than WT effector CD8$^+$ T cells (Figure 4E). *Menin* KO CD8$^+$ T cells expressing

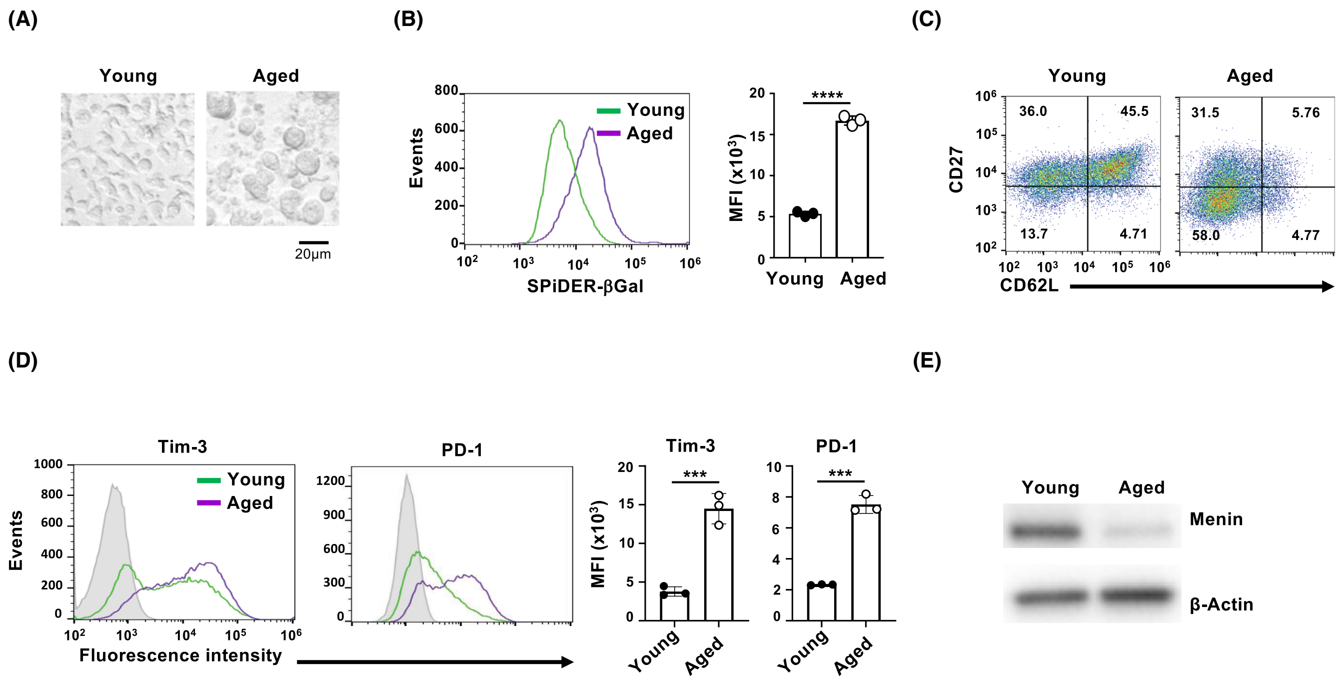

FIGURE 3 CD8$^+$ T cells from aged mice show a senescent-T cell like phenotype. CD8$^+$ T cells were prepared from young (6–10 weeks) and aged (≥30 weeks) mice. The cells were stimulated with anti-TCR-β mAb plus anti-CD28 mAb with IL-2 for 2 days, and then further expanded with IL-2 for the indicated days. (A) The results of cellular morphology of CD8$^+$ T cells was measured on day 12 after initial stimulation in vitro. (B) The expression of SA-βgal in CD8$^+$ T cells was measured on day 12 (n = 3 biological replicates). (C) The surface staining profile of CD27 and CD62L on the cells on day 7. (D) Representative results of surface staining of Tim-3 and PD-1 in the cells on day 7 (n = 3 biological replicates). (E) The results of immunoblotting of Menin in the cells on day 7. The amount of β-Actin was used as the loading control. The results of (A–E) are representative of at least three independent experiments with similar results. The error bar displayed indicates the mean ± SD (B and D). ***P < 0.001, ****P < 0.0001 (two-tailed unpaired t-test).

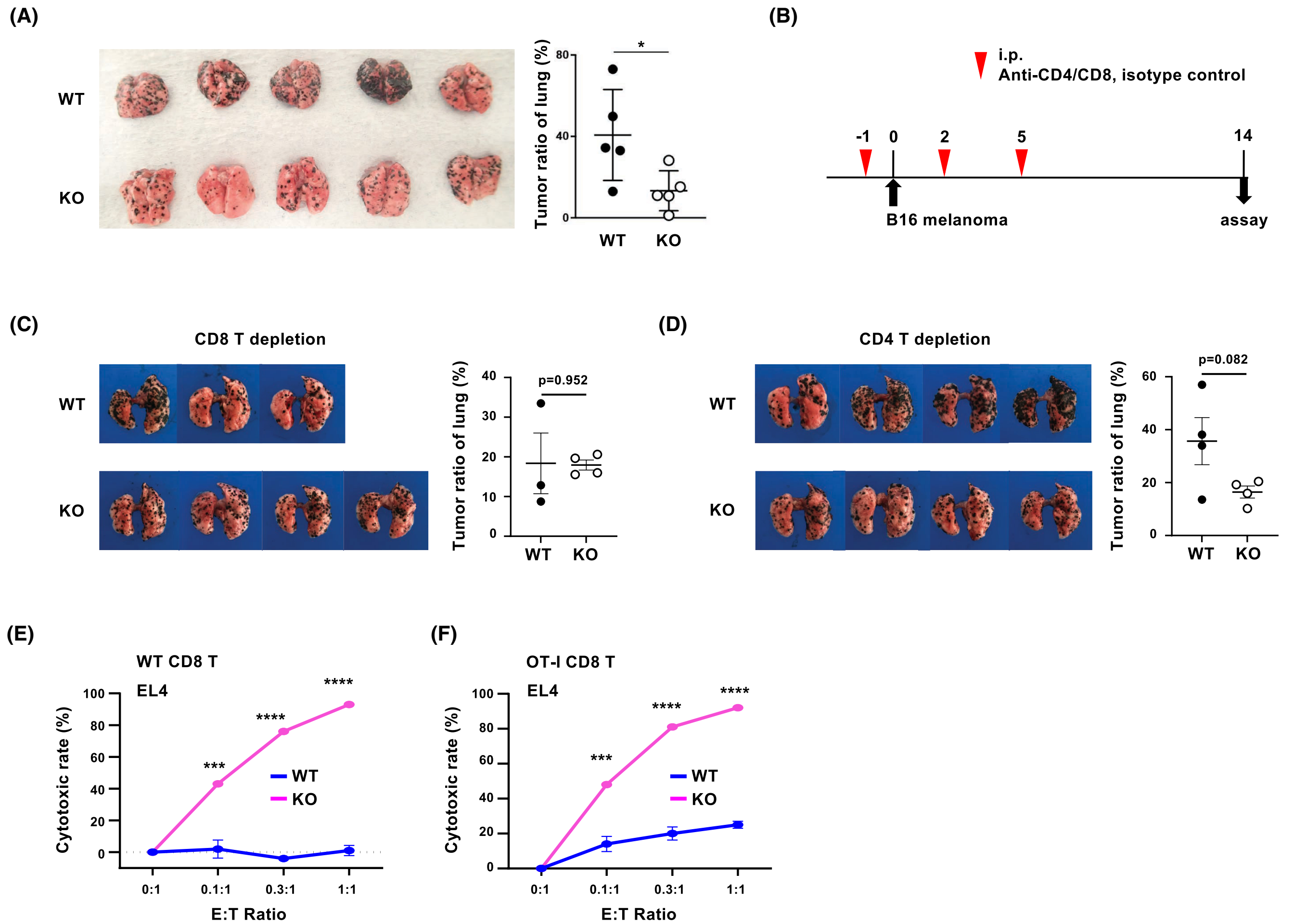

FIGURE 4 Increased antitumor activity in *Menin* KO mice and CD8$^+$ T cells. (A) The amounts of B16 lung metastasis on day 14 after tumor inoculation in WT mice and *Menin* KO mice ($n=5$ per group). (B) Experimental design of the B16 lung metastasis model and depletion of CD4$^+$ T cells or CD8$^+$ T cells in vivo. (C) Depletion of CD8$^+$ T cells in WT ($n=3$ per group) and *Menin* KO mice ($n=4$ per group). (D) Depletion of CD4$^+$ T cells in WT and *Menin* KO mice ($n=4$ per group). (E) Naïve CD8$^+$ T cells from WT or *Menin* KO mice were stimulated with anti-TCR-β mAb plus anti-CD28 mAb with IL-2 for 2 days, and the cells were then further expanded with IL-2 for an additional 5 days. The results of the killing assay of WT and *Menin* KO CD8$^+$ T cells on day 7. (F) The results of the killing assay of WT and *Menin* KO OT-1 CD8$^+$ T cells on day 7. The results of (E) and (F) are based on triplicate wells of a single representative experiment. The results are representative results of three independent experiments with similar results. The error bar displayed indicates the mean ± SEM (A and C–F). *$P<0.05$, ***$P<0.001$, ****$P<0.0001$ (two-tailed unpaired *t*-test).

the OVA-specific OT-1 TCR also showed increased antitumor activity (Figure 4F), indicating that *Menin* KO effector CD8$^+$ T cells have enhanced NK cell-like innate cytotoxic activity.

3.5 | Menin restricts the expression of cytotoxic molecules in effector CD8$^+$ T cells

Next, we used RNA-seq to analyze the gene expression profile of in vitro-generated *Menin* KO effector CD8$^+$ T cells. RNA-seq revealed that the mRNA expression of effector CD8$^+$ T cell-related molecules was increased in *Menin* KO effector CD8$^+$ T cells in comparison to that in WT CD8$^+$ T cells (Figure 5A). The ratio of IFN-γ- and TNF-α-producing cells in *Menin* KO effector CD8$^+$ T cells was comparable to that in WT CD8$^+$ T cells (Figure 5B). The mRNA level of *perforin1* in *Menin* KO CD8$^+$ T cells was higher than that in WT CD8$^+$ T cells (Figure 5C). The expression of granzyme A, granzyme B, and granzyme C was increased in *Menin* KO CD8$^+$ T cells (Figure 5D). These results suggest that Menin limits the effector function of CD8$^+$ T cells. We and other groups have reported that Menin binds to the enhancer regions at target gene loci and regulates the gene expression.[19,38] Therefore, we performed ChIP sequencing (ChIP-seq) to assess the binding of Menin to the granzyme gene loci and the histone H3K27 acetylation status, an active enhancer marker. The specific binding of Menin to the 5′ upstream region of the *perforin1*, *granzyme a*, *granzyme b*, and *granzyme c* gene loci were detected in effector CD8$^+$ T cells (Figure 5E, upper panel). The level of histone H3K27 acetylation at the *perforin1*, *granzyme a*, and *granzyme c* gene loci, but not *granzyme b* was increased in *Menin* KO CD8$^+$ T cells (Figure 5E, lower panel).

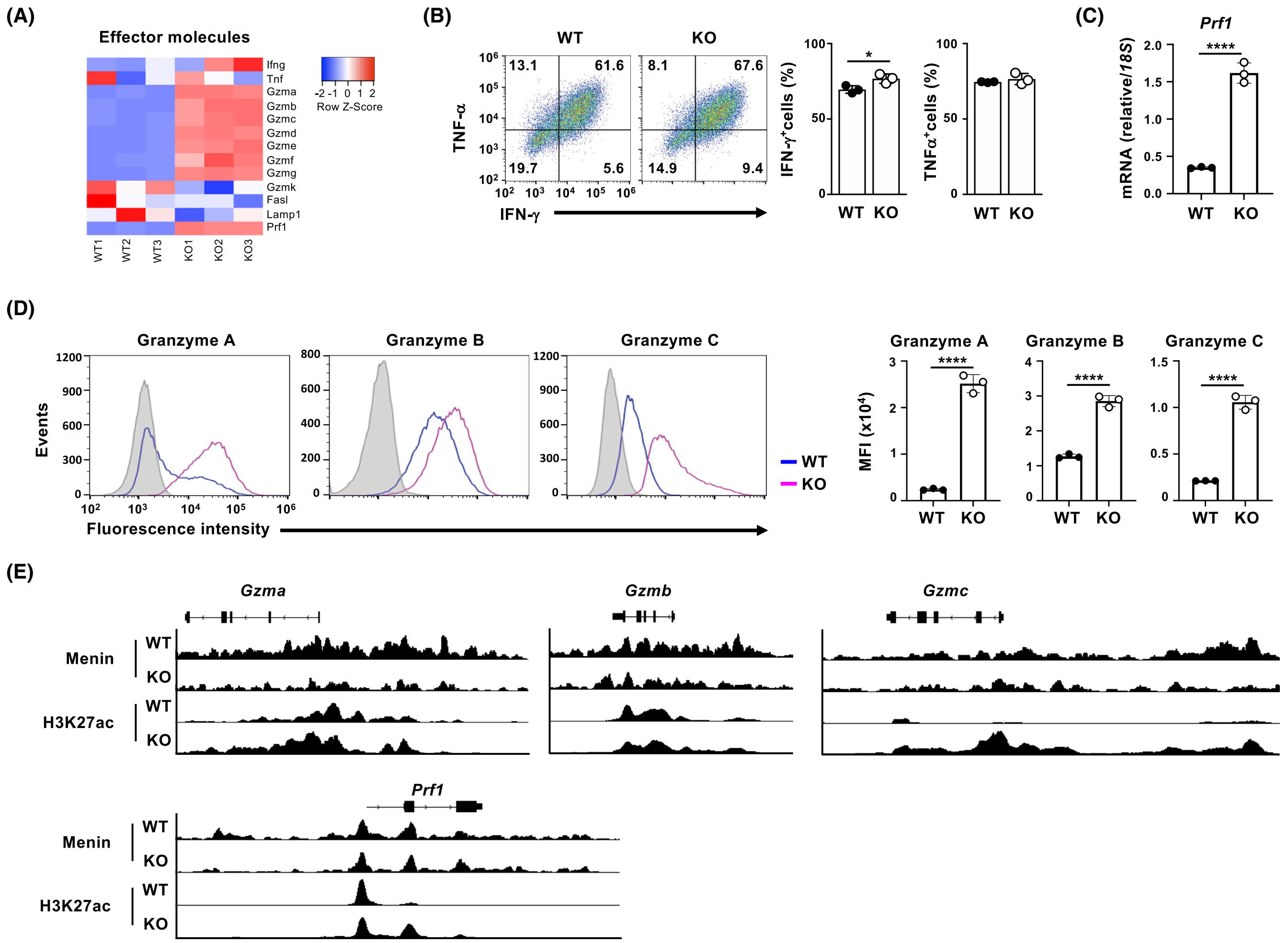

FIGURE 5 *Menin* KO CD8$^+$ T cells have higher cytotoxic activity. Naïve CD8$^+$ T cells from WT and *Menin* KO mice were stimulated and cultured in as Figure 4. (A) The result of RNA-seq analyses of WT and *Menin* KO CD8$^+$ T cells on day 7 ($n = 3$ biological replicates). A heatmap of the expression of CD8-effector related genes. (B) Representative results of intracellular staining of IFN-γ and TNF-α in the cells on day 7 ($n = 3$ biological replicates). (C) The results of the *prf1* mRNA expression in WT and *Menin* KO CD8$^+$ T cells on day 7 as determined by quantitative RT-PCR ($n = 3$ technical replicates). (D) Representative results of intracellular staining of GzmA, GzmB and GzmC in the cells on day 7. (E) The results of Menin binding and the levels of histone H3K27ac at the *gzma*, *gzmb*, *gzmc*, and *prf1* gene loci in the cells on day 10 were determined using ChIP sequencing. The results of (B–D) are representative result of three independent experiments. The error bar displayed indicates the mean ± SD (B–D). *$P < 0.05$, ****$P < 0.0001$ (two-tailed unpaired *t*-test).

3.6 | Menin restricts the expression of NK-cell-related molecules in effector CD8$^+$ T cells

RNA-seq demonstrated that the expression of NK cell-associated surface molecules was higher in *Menin* KO effector CD8$^+$ T cells than in WT CD8$^+$ T cells (Figure 6A). The high expression of *Cd244*, *Ncr1*, *Klrb1c* (*Nk1.1*), *Fcer1g*, *Fcgr3*, and *Tyrobp* (*Dap12*) in *Menin* KO effector CD8$^+$ T cells was confirmed by qRT-PCR (Figure 6B). Flow cytometry showed that CD244 and NK1.1 were expressed on all Menin KO effector CD8$^+$ T cells, with higher expression levels than on WT effector CD8$^+$ T cells (Figure 6C). Furthermore, FcεR1γ-poitive cells were increased in *Menin* KO CD8$^+$ T-cell culture in comparison to that in WT CD8$^+$ T-cell culture (Figure 6D). The specific binding of Menin to the *Cd244*, *Klrb1c*, and *Fcer1g* gene loci was detected by ChIP-seq (Figure 6E, upper panel). The level of histone H3K27 acetylation of the *Cd244*, *Klrb1c*, and *Fcer1g* gene loci was increased in *Menin* KO CD8$^+$ T cells (Figure 6E, lower panel). These results suggest that Menin negatively regulates enhancer activity and limits the expression of cytotoxic molecules and NK-cell related molecules in effector CD8$^+$ T cells.

4 | DISCUSSION

In this paper, our findings show that senescent CD8$^+$ T cells may play a role in antitumor immunity in the elderly by acquiring NK cell-like innate immune functions. We show that CD8$^+$ T cells from aged mice exhibit NK cell-like antigen-independent cytotoxic activity, accompanied by increased expression of cytotoxic granules and NK receptors after activation in vitro. Furthermore, CD8$^+$ T cells from

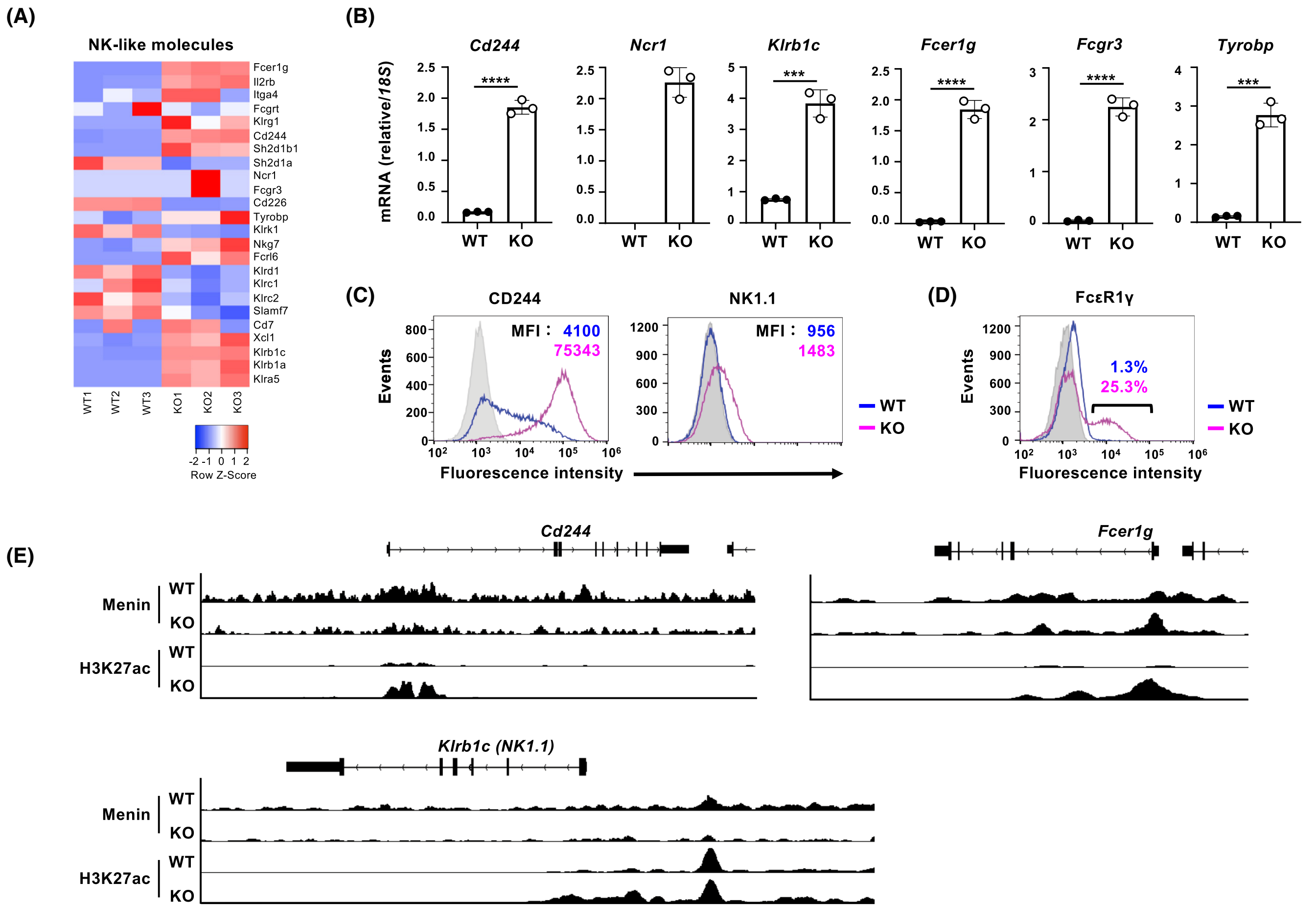

FIGURE 6 The increased expression of NK-related molecules in *Menin* KO CD8$^+$ T cells and control by the epigenetic reduction of Menin. (A) A heatmap of the expression of CD8-effector related genes in (Figure 5A) cells ($n=3$ biological replicates). (B) The mRNA expression of NK-related genes in WT and *Menin* KO CD8$^+$ T cells on day 7 was analyzed by quantitative RT-PCR. The results are presented relative to the expression of *18s* rRNA with the standard deviation (mean ± SD, $n=3$ technical replicates). (C) Representative staining profile of CD244 and NK1.1 in WT and *Menin* KO CD8$^+$ T cells on day 7. The median fluorescence intensity (MFI) is shown. (D) Representative staining profile of FcεR1γ in WT and *Menin* KO CD8$^+$ T cells on day 7. The percentages of FcεR1γ$^+$ cells are shown. (E) The results of the Menin binding and the levels of histone H3K27 acetylation at *Cd244*, *FcεR1γ*, and *Klrb1c* gene loci in the WT and *Menin* KO CD8$^+$ T cells on day 10 were determined using ChIP-seq. The results of (B–D) are representative of at least three independent experiments with similar results. ***$P<0.001$, ****$P<0.0001$ (two-tailed unpaired *t*-test).

aged mice exhibit a senescence-like phenotype and morphology, with increased SA-βgal activity. We have previously reported that *Menin*-deficient CD8$^+$ T cells show early senescence after activation in vitro.[20,21] In this study, we found that the expression of Menin in in vitro-generated effector CD8$^+$ T cells from aged mice is markedly decreased in comparison to that from young mice. Like effector CD8$^+$ T cells from aged mice, *Menin* KO effector CD8$^+$ T cells induced *in vitro* showed the increased expression of cytotoxic proteins, such as perforin and granzymes, NK cell-related cell surface molecules. In addition, *Menin* KO effector CD8$^+$ T cells exhibited NK cell-like antigen-independent cytotoxic activity. These results suggest that effector CD8$^+$ T cells from aged mice acquire NK cell-like innate functions through the downregulation of Menin.

In the present study, it is not clear why the expression of Menin is reduced when CD8$^+$ T cells from aged mice are activated. We have observed that the protein expression of Menin is strongly downregulated in effector CD8$^+$ T cells from aged mice, whereas the expression of Menin mRNA showed no reduction (J.S., and M.Y. unpublished observation). These results indicate that the expression of Menin protein may be regulated by post-transcriptional machinery. Several microRNA have been reported to be upregulated in terminally differentiated CD8$^+$ T cells.[39] Among them, miR-24 has been reported to inhibit the translation of Menin in BON1 cells (a human cell line derived from pancreatic neuroendocrine tumor).[40] Although it remains to be confirmed whether miR-24 is actually involved in the regulation of the Menin expression in senescent CD8$^+$ T cells, studies to identify senescent-associated microRNA may become important not only for studying T-cell senescence, but also for the analysis of the innate lymphoid cell-like T-cell innate immune response.

Menin is a component molecule of the Mixed-linage leukemia 1 (MLL1) and MLL2 complexes with histone H3K4 methyltransferase activity.[25] The MLL1/MLL2 complexes are thought to preserve the gene expression through the maintenance of histone H3K4 tri- and di-methylation of the active promoter region.[41–43] In contrast, Menin also forms a complex with HDACs and suppresses the gene expression. In this paper, we show the binding of Menin to the NK cell related gene loci, such as *perforin1*, *granzyme a*, *granzyme b*, and *granzyme c*, *Cd244*, *Klrb1c*, and *Fcer1g*. An increased level of histone H3K27 acetylation at those gene loci was detected in *Menin* KO effector $CD8^+$ T cells. We previously reported that the increased expression of senescent-associated secretary phenotype (SASP)-related gene in *Menin* KO effector $CD8^+$ T cells was restored by deletion of the *Utx*, a histone H3K27 demethylase.[21] Utx has been reported to be a component molecule of the MLL3 and MLL4 complexes.[44,45] The MLL3 and MLL4 complexes have been reported to not only demethylate histone H3K27 at the inactive and bivalent enhancer region, but also acetylate via histone acetyltransferase in the complexes.[46] Therefore, it is likely that Menin associates with HDACs and inhibits the NK cell-related innate gene expression in effector $CD8^+$ T cells via repression of enhancer activity. The loss of Menin in senescent $CD8^+$ T cells resulted in the enhanced expression of NK cell-related genes and the subsequent acquisition of NK cell-like innate functions, (e.g., antigen-independent cytotoxic activity).

In the present study, RNA-seq revealed that the expression of NK receptors was increased in senescent $CD8^+$ T cells and *Menin* KO effector $CD8^+$ T cells. The expression of adaptor molecules that transduce activation signals was also upregulated, such as FcεR1γ. However, it is still unclear which NK receptors and adaptor molecules are important in the antigen-independent innate cytotoxic activity of senescent $CD8^+$ T cells. Recently, a population of FcεR1γ-positive innate immunocompetent $CD8^+$ T cells called innate-like T cells with high cytotoxic potential (ILTCK) was identified and reported to be responsible for antitumor immunity via antigen-independent cytotoxic activity.[47] We have shown that some senescent $CD8^+$ T cells and *Menin* KO $CD8^+$ T cells express FcεR1γ. Thus, FcεR1γ-positive cells may be important for antigen-independent cytotoxic activity in senescent $CD8^+$ T cells. Furthermore, we demonstrated that $CD8^+$ T cells that moderately expressed asialo GM1 were increased in the spleen and lungs of aged mice compared to young mice (Figure S1). Asialo $GM1^+CD8^+$ T cells reportedly proliferate and produce IFN-γ in response to IL-12 plus IL-2[48]. Furthermore, asialo $GM1^+CD8^+$ T cells co-expressed IL-18 receptor (M.Y. and J.S., unpublished observation). Given these findings, the asialo $GM1^+CD8^+$-T cell population may include senescent $CD8^+$ T cells. These cells are activated by cytokines (i.e., IL-12, IL-18, and IL-2) and exert antitumor effects against various types of tumor cells in an antigen-independent manner.

Our findings indicate that senescent $CD8^+$ T cells may support immune responses by acquiring NK cell-like innate immune functions in elderly individuals with reduced numbers of naïve $CD8^+$ T cells and TCR repertoires.

AUTHOR CONTRIBUTIONS

Toshio Kakuda, Junpei Suzuki, Tadahiko Kikugawa, Takashi Saika, and Masakatsu Yamashita contributed to the conception and design of this study. Toshio Kakuda, Junpei Suzuki, and Yuko Matsuoka conducted the experiments and analyzed the data. Toshio Kakuda and Masakatsu Yamashita drafted and revised the manuscript. Junpei Suzuki and Masakatsu Yamashita made important revisions to the manuscript. The final manuscript was read and approved by all authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in association with the present study.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: The animal experiments were approved by the Ehime University Animal Experiment Committee (approval no. 05KO20-1,16) and conducted in accordance with the laws and regulations concerning animal experiments, animal care and keeping standards, and basic guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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