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SCD1 inhibition enhances the effector functions of CD8$^+$ T cells via ACAT1-dependent reduction of esterified cholesterol

Toshihiro Sugi[1] | Yuki Katoh[2,3] | Toshikatsu Ikeda[2] | Daichi Seta[4] | Takashi Iwata[3] | Hiroshi Nishio[3] | Masaki Sugawara[3] | Daiki Kato[5] | Kanoko Katoh[1] | Kei Kawana[1] | Tomonori Yaguchi[6] | Yutaka Kawakami[7] | Shuichi Hirai[2]

[1]Department of Obstetrics and Gynecology, Nihon University School of Medicine, Tokyo, Japan

[2]Division of Anatomical Science, Department of Functional Morphology, Nihon University School of Medicine, Tokyo, Japan

[3]Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan

[4]Nihon University School of Medicine, Tokyo, Japan

[5]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

[6]Department of Immunology and Genomic Medicine, Center for Cancer Immunotherapy and Immunobiology, Kyoto University Graduate School of Medicine, Kyoto, Japan

[7]Department of Immunology, School of Medicine, International University of Health and Welfare, Chiba, Japan

Correspondence
Yuki Katoh, Division of Anatomical Science, Department of Functional Morphology, Nihon University School of Medicine, 30-1 Oyaguchi-kami-cho, Itabashi-ku, Tokyo 173-0032, Japan and Department of Obstetrics and Gynecology, Keio University School of Medicine, 35 Shinano-machi, Shinjuku-ku, Tokyo 160-8582, Japan.
Email: kato.yuki@nihon-u.ac.jp; yukikatoh@keio.jp

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Abstract

We previously reported that the inhibition of stearoyl-CoA desaturase 1 (SCD1) enhances the antitumor function of CD8$^+$ T cells indirectly via restoring production of DC recruiting chemokines by cancer cells and subsequent induction of antitumor CD8$^+$ T cells. In this study, we investigated the molecular mechanism of direct enhancing effects of SCD1 inhibitors on CD8$^+$ T cells. In vitro treatment of CD8$^+$ T cells with SCD1 inhibitors enhanced IFN-γ production and cytotoxic activity of T cells along with decreased oleic acid and esterified cholesterol, which is generated by cholesterol esterase, acetyl-CoA acetyltransferase 1 (ACAT1), in CD8$^+$ T cells. The addition of oleic acid or cholesteryl oleate reversed the enhanced functions of CD8$^+$ T cells treated with SCD1 inhibitors. Systemic administration of SCD1 inhibitor to MCA205 tumor-bearing mice enhanced IFN-γ production of tumor-infiltrating CD8$^+$ T cells, in which oleic acid and esterified cholesterol, but not cholesterol, were decreased. These results indicated that SCD1 suppressed effector functions of CD8$^+$ T cells through the increased esterified cholesterol in an ACAT1-dependent manner, and SCD1 inhibition enhanced T cell activity directly through decreased esterified

Abbreviations: ACAT1, acetyl-CoA acetyltransferase 1; CAR, chimeric antigen receptor; CCL4, C–C motif chemokine ligand 4; ChO, cholesteryl oleate; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cell; ER, endoplasmic reticulum; GaLV, Gibbon ape leukemia virus; GC–MS, gas chromatography–mass spectrometry; GPC1, glypican-1; GPC3, glypican-3; GzmB, granzyme B; IFN, interferon; IL-2, interleukin-2; MACS, magnetic activated cell sorting; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; PPAR, peroxisome proliferator-activated receptor; SCD1, stearoyl-CoA desaturase 1; scFv, single-chain variable fragment; SREBP1, sterol regulatory element-binding transcription factor 1; sXBP1, spliced x-box binding protein 1; TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte; TNF-α, tumor necrosis factor-α; Wnt, wingless-related integration site; WST-1, water-soluble tetrazolium 1; γCD, γ-cyclodextrin.

Toshihiro Sugi and Yuki Katoh contributed equally to this work.

cholesterol. Finally, SCD1 inhibitors or ACAT1 inhibitors synergistically enhanced the antitumor effects of anti-PD-1 antibody therapy or CAR-T cell therapy in mouse tumor models. Therefore, the SCD1–ACAT1 axis is regulating effector functions of $CD8^+$ T cells, and SCD1 inhibitors, and ACAT1 inhibitors are attractive drugs for cancer immunotherapy.

KEYWORDS
acetyl-CoA acetyltransferase 1 (ACAT1), $CD8^+$ T cell, esterified cholesterol, oleic acid, stearoyl-CoA desaturase 1 (SCD1)

1 | INTRODUCTION

Cancer immunotherapies, such as immune checkpoint inhibitors including antibodies against PD-1/PD-L1 and CTLA-4, and CAR-T cell therapy, have shown durable clinical effects in patients with various cancers.[1–5] However, their efficacy remains limited to only a subset of patients.[6] The pretreatment immune status of the tumor microenvironment varies among cancer patients and correlates with their prognosis and response to cancer therapies.[6,7] In particular, good clinical responses to immune checkpoint inhibitors have been observed in patients with T cell-inflamed tumors harboring a high mutational load.[8] Therefore, further analyses of cancer immunopathology and the development of novel immune-modulating strategies, which convert non-T cell-inflamed tumors into T cell-inflamed tumors, are required.[6,9]

SCD1 is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids, such as palmitoleic and oleic acids, from saturated fatty acids such as palmitic and stearic acids.[10,11] In a previous study, we found that SCD1 has an immunosuppressive activity in cancer cells and immune cells.[12] We also reported that SCD1 inhibition positively regulates the cancer immune cycle and improves the therapeutic effect of anti-PD-1 antibodies by modulating the Wnt/β-catenin pathway in cancer cells or ER stress pathway in $CD8^+$ T cells.[12] Furthermore, SCD1 inhibitors act directly on $CD8^+$ T cells to enhance antitumor function.[12] However, the detailed molecular mechanism by which SCD1 inhibitors directly enhance the antitumor activity of $CD8^+$ T cells remains to be elucidated.

The ER enzyme ACAT1 is a cholesterol-metabolizing enzyme that transfers long-chain fatty acids to cholesterol to form cholesteryl esters.[13,14] ACAT1 has recently attracted attention as a target for cancer therapy. Inhibition of ACAT1 has been shown to effectively reduce hepatocellular tumors,[15] inhibit pancreatic tumor growth and metastasis,[16] and prevent prostate cancer[17] in mouse models. Furthermore, inhibition of ACAT1 in $CD8^+$ T cells enhances antitumor immune responses and potentiates the effects of cancer immunotherapy.[18] Interestingly, a relationship between SCD1 and ACAT1 has been reported, whereby inhibition or knockdown of ACAT1 suppresses SREBP1, thereby downregulating SCD1 and inhibiting prostate cancer progression.[19] However, the mechanism by which ACAT1 is regulated as a downstream factor of SCD1 in $CD8^+$ T cells remains unclear.

In this study, we investigated the mechanism by which SCD1 inhibitors directly enhance the antitumor activity of $CD8^+$ T cells by focusing on the crosstalk mechanism between SCD1 and ACAT1, and the involvement of the ACAT1 metabolite esterified cholesterol.

2 | MATERIALS AND METHODS

2.1 | Cells and reagents

The mouse sarcoma cell line MCA205 and human cervical cancer cell line HeLa were purchased from the American Type Culture Collection. Cell lines were verified to be negative for mycoplasma contamination using TaKaRa PCR Mycoplasma Detection Set prior to experiments. MCA205 cell was transduced with lentivirus vectors encoding mouse GPC1 (mGPC1) cDNA as previously described.[20] MCA205 stably expressing mGPC1 are designated as MCA205-mGPC1. Human $CD8^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) of consenting healthy volunteers by MACS (Miltenyi: #130-045-201). Human naïve $CD8^+$ T cells were defined as $CD8^+CD45RO^-CD244^-CD56^-CD57^-$ cells and were isolated from PBMCs by MACS (Miltenyi: #130-093-244). Human memory $CD8^+$ T cells were defined as $CD8^+CD45RO^+CD45RA^-CD56^-CD57^-$ cells and were isolated from PBMCs by MACS (Miltenyi: #130-094-412). Mouse $CD8^+$ T cells were isolated from tumor tissue by MACS (Miltenyi: #130-116-478) on day 20 after tumor transplantation. SCD1 inhibitors A939572 and CAY10566 were purchased from APExBIO and Cayman, respectively. ACAT1 inhibitors Avasimibe and CP-113818 were purchased from MedChemExpress. Recombinant human IL-2 was purchased from Novartis. ELISA kits for human IFN-γ (#555142) and TNF-α (#555212), and mouse IFN-γ (#555138) were purchased from BD Biosciences. ELISA kit for human GzmB was purchased from Bio-Techne (#DY2906-05).

2.2 | Cell culture

Human $CD8^+$ T cells were cultured in AIM-V medium (Thermo Fisher Scientific) containing 10% heat-inactivated human AB serum and 300 IU/mL recombinant human IL-2. Mouse $CD8^+$ T cells and mouse cancer cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Mouse CAR-T cells were cultured in RPMI1640 medium

(Thermo Fischer Scientific) containing 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 10 mM I-HEPES (Thermo Fischer Scientific), and 50 IU/mL of IL-2 (Novartis). The concentration of serum in experiments with SCD1 inhibitors was 2%.

2.3 | In vitro CD8$^+$ T cell activation

Human CD8$^+$ T cells were activated by an anti-CD3 monoclonal antibody (OKT3, 2 mg/mL), anti-CD28 mAb (9.3, 2 mg/mL), and IL-2. SCD and ACAT1 inhibitors were added at 2 days after stimulation. Samples for ELISA were collected on day 4, and samples for GC–MS, cholesterol assays, western blotting, and qPCR were collected on day 5.

2.4 | GC–MS analysis

Extraction of fatty acid fractions from human CD8$^+$ T cells (1×10^6), mouse tumor-infiltrating CD8$^+$ T cells (1×10^5) and tumor tissue samples were according to the previous description.[12] GC–MS analysis was performed on a Shimadzu GC–MS QP2010 Ultra equipped with an AOC20i autoinjector and Rtx-5MS column (30 m, 0.25 mm, 0.25 μm df) in 70 eV electron ionization mode. The temperature program for GC–MS was as previously described.[12] Selected ion monitoring for quantification was performed by recording ions at *m/z* 311.20 for palmitoleic acid-trimethylsilyl derivative, *m/z* 327.20 for margaric acid-trimethylsilyl derivative, and *m/z* 339.20 for oleic acid-trimethylsilyl derivative.

2.5 | Flow cytometry

For human CD8$^+$ T cell staining, cells were stained with anti-TIGIT (PE; BioLegend, clone: A15153G), CD8 (Alexa700; BioLegend, clone: RPA-T8) and 4-1BB (BV 421; BD Biosciences, clone: 4B4-1). Flow cytometry sample acquisition was performed using a Gallios flow cytometer (Beckman Coulter) and analysis was performed using Kaluza software (Beckman Coulter).

2.6 | RNA extraction and RT-qPCR analysis

Total RNA was isolated from cultured cells recovered in vitro using an RNeasy Mini Kit (Qiagen). RT-qPCR was performed by the standard $2^{-\Delta C_t}$ method. The relative quantification value is expressed as $2^{-\Delta C_t}$, where ΔC_t is the difference between the mean C_t value of triplicate measurements of the sample and the endogenous GAPDH control. TaqMan RT-PCR primers and probes for human ACAT1 and ELOVL1-6 were purchased from Integrated DNA Technologies. SYBR Green RT-PCR primers for human sXBP1 were purchased from Sigma-Aldrich.

2.7 | Western blotting

Cytoplasmic samples were prepared using RIPA buffer (Thermo Fisher Scientific) containing cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche) in accordance with the manufacturer's instructions. Cell lysates were centrifuged at $14{,}000\times g$ for 30 min at 4°C. The protein concentration of each sample was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad) with bovine serum albumin (Bio-Rad) as the standard. Western blotting was performed according to the standard protocol. Antibodies used in western blotting were rabbit anti-ACAT1 (EPR10359; Abcam) or rabbit anti-GAPDH (FL-335; Santa Cruz Biotechnology) antibodies.

2.8 | Cholesterol assay

Cholesterol and esterified cholesterol levels in CD8$^+$ T cells were evaluated using a cholesterol assay kit (K603-100; BioVision). Briefly, cholesterol was extracted from human and mouse CD8$^+$ T cells in accordance with the manufacturer's instructions. Then, changes in the cholesterol concentration after SCD1 inhibitor treatment were determined. A colorimetric assay was conducted at an optical density of 570 nm to determine the cholesterol content from a standard curve.

2.9 | Preparation of the ChO/γCD inclusion complexes

ChO (0.05 mmol)/γ-CD (1.5 mmol) nanoparticles were prepared by the solvent diffusion method in which ethanol containing ChO was added to a γ-CD aqueous solution.[21,22]

2.10 | WST-1 assay

CD8$^+$ T cell viability was evaluated using WST-1 Solution Reagent (Roche) according to the manufacturer's instructions.

2.11 | Tumor-bearing mouse model

Mice were bred at the animal facilities of Nihon University in accordance with the guidelines for animal experimentation. Mice were maintained in a specific-pathogen-free environment with a 12 h light–dark cycle (dark cycle occurring from 8:00 p.m. to 8:00 a.m.). Six- to 8-week-old C57B/6 mice were subcutaneously injected with 5×10^5 MCA205 cells in their flank on day 0. On day 4, mice were treated with the vehicle (50% v/v polyethylene glycol 400, 20% v/v propylene glycol, 20% v/v vitamin E, 5% v/v ethanol, and 5% w/v polyvinylpyrrolidone) or 10 mg/kg SCD1 inhibitor (A939572). Mice received the vehicle or inhibitor via oral gavage twice daily for 16 days.

For Avasimibe treatment, on day 4, mice were treated with the vehicle (PBS) or 15 mg/kg ACAT1 inhibitor (Avasimibe). Mice received the vehicle or inhibitor via oral gavage once daily for 16 days. Mice in the combined therapy group also received anti-PD-1 or isotype-matched antibodies (200 μg/mouse; Bio X Cell) on days 4, 7, and 10. The tumor volume was calculated by direct tumor measurements every 4 days using the following formula: 1/2 [length × (width)^2].

2.12 | Experiments with CAR-T cells

2.12.1 | Generation of GPC1-specific murine CAR-T (GPC1-mCAR-T) cells and GPC3-specific human CAR-T (GPC3-hCAR-T) cells

GPC1-specific murine CAR-T cells and GPC3-specific human CAR-T cells were generated according to previous descriptions.[23] Human GPC3 CAR plasmid was generated by replacing VL and VH regions in scFv of our previously generated human GPC1 CAR plasmid[23] with those of anti-GPC3 mAb (clone: GC33; Table S1).[24,25] After 12 h, supernatants were replaced by fresh medium and retroviral supernatants were collected at 24 h after replacement of medium. This retroviral supernatant was centrifuged onto RetroNectin (Takara Bio Inc) coated plates at 2000 × *g* for 2 h at 32°C.

For the activation of murine T cells, splenocytes were collected from C57BL6 mice and activated with 2.5 mg/mL concanavalin A (Sigma-Aldrich) supplemented with 1 ng/mL rmIL-7 (Peprotech) for 1 day before transduction. The stimulated cells were then transduced by spin-down onto the retrovirus plates for 10 min at 1000 × *g*. For human T cells, PBMCs from healthy donors were stimulated with soluble 50 ng/mL OKT-3 (Thermo Fischer Scientific) for 2 days before transduction. The stimulated cells were then transduced by spin-down onto the retrovirus plates for 10 min at 1000 × *g*.

2.12.2 | Cytokine secretion assay

GPC1-mCAR-T cells (1×10^5 cells/well) were co-cultured with MCA205-mGPC1 cells (5×10^4 cells/well) in a 96-well plate and supernatants were harvested after 24 h. Mouse IFN-γ was measured by ELISA (BD Biosciences).

2.12.3 | Chromium release assay

MCA205-mGPC1 cells (target cells) were loaded with Cr^{51} (Japan Radioisotope Association) and co-cultured with differing amounts of GPC1-mCAR-T cells. After a 4 h incubation at 37°C, the release of free Cr^{51} was measured by TopCount NXT (PerkinElmer Inc) as previously described.[20] The percent-specific lysis was calculated using the formula: % specific lysis = 100 × (experimental cpm release − spontaneous cpm release)/(total cpm release − spontaneous cpm release).

2.12.4 | Mouse model

Mice were bred at the animal facilities of Keio University in accordance with the guidelines for animal experimentation. In total, 5×10^5 MCA205-mGPC1 cells were subcutaneously inoculated into the flank of 6–8-week-old C57BL/6 mice and conditioned for adoptive cell therapies, as previously reported.[26,27] On days 2–3, the mice were conditioned with 2.5 Gy total body irradiation immediately before the T cell transfer and the cultured mCAR-T cells or mouse control T (mCont-T) cells (5×10^6 cells per mouse) were intravenously administered. Subsequently, the mice were given intraperitoneal injections of 50,000 IU/mouse rhIL-2 twice daily up to six doses. Mice in the combined therapy group also received 10 mg/kg A939572 via oral gavage twice daily for 16 days, starting from day 4 after tumor transplantation.

2.13 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 software. Comparisons between two groups were made using unpaired or paired (for matched comparisons) two-tailed Student's *t*-tests or the non-parametric Mann–Whitney *U*-test. Multiple comparisons were made by one-way ANOVA with Tukey's multiple comparisons test. Data are presented as means ± SD. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | SCD1 inhibitors enhanced IFN-γ production by human CD8$^+$ T cells through decrease of intracellular oleic acid

We have previously demonstrated that SCD1 has a direct immunosuppressive activity on CD8$^+$ T cells, but the molecular mechanism has not been clarified.[12] Therefore, we evaluated whether SCD1 inhibitors act directly on CD8$^+$ T cells and enhance their antitumor activity. Treatment of human CD8$^+$ T cells activated in vitro with anti-CD3 and anti-CD28 mAbs with SCD1 inhibitors, A939572 or CAY10566, resulted in the reduction of intracellular levels of oleic acid and palmitoleic acid, which are fatty acids generated by SCD1 (Figure 1A,B), and significantly enhanced IFN-γ production by CD8$^+$ T cells (Figure 1C). This enhancement of IFN-γ production by SCD1 inhibitors was canceled by the addition of oleic acid, but not palmitoleic acid (Figure 1C). Since palmitoleic acid may be metabolized to oleic acid by Elovl6,[28] we evaluated the intracellular oleic acid level when palmitoleic acid was added in human CD8$^+$ T cell culture, and found no increase of oleic acid. Furthermore, the expression of Elovl6 in human CD8$^+$ T cells was significantly low and the oleic acid level did not change (Figure S1A,B). SCD1 inhibitors also enhanced cell proliferation of CD8$^+$ T cells, consistent with our previous report,[12] but this effect was not canceled by the addition of oleic and palmitoleic acids (Figure 1D). Furthermore, SCD1 inhibitors enhanced the expression of

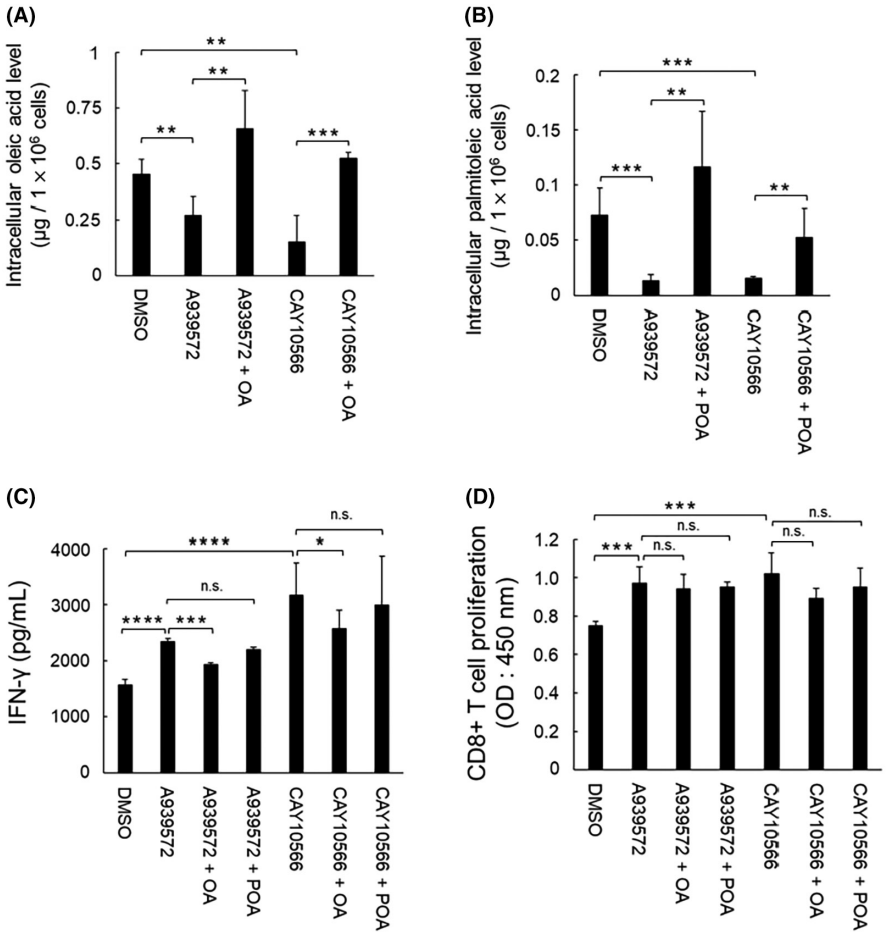

FIGURE 1 SCD1 inhibitors enhance the activation of human CD8$^+$ T cells. CD8$^+$ T cells were isolated from human PBMCs using MACS and activated with an anti-CD3 monoclonal antibody (mAb), anti-CD28 mAb, and IL-2. CD8$^+$ T cells were treated with dimethylsulfoxide (DMSO), SCD1 inhibitors (A939572 and CAY10566), SCD1 inhibitors + oleic acid (OA), or SCD1 inhibitors + palmitoleic acid (POA). (A) Intracellular oleic acid levels in human CD8$^+$ T cells. (B) Intracellular palmitoleic acid levels in human CD8$^+$ T cells. (C) IFN-γ levels in culture supernatants of CD8$^+$ T cells were measured using ELISA. (D) Cell proliferation of CD8$^+$ T cells was evaluated by WST-1 assay. Data are expressed as means ± SD ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. n.s., not significant.

4-1BB and TIGIT in CD8$^+$ T cells and also enhanced the effector function of both naïve and memory CD8$^+$ T cells (Figure S2A–C). These results suggest that SCD1 suppresses the effector function of CD8$^+$ T cells and in particular, that the suppression of IFN-γ production involves an SCD1-dependent increase in intracellular oleic acid.

3.2 | SCD1 inhibitors enhanced human CD8$^+$ T cell activation in an ACAT1 dependent manner

We then attempted to clarify the relationship between SCD1 and ACAT1 in the CD8$^+$ T cell effector functions, because cholesterol esterase ACAT1 gene knockout was reported to enhance cytokine production by CD8$^+$ T cells[18] and oleic acid is the preferential substrate of ACAT1.[29] Although ACAT1 inhibitors Avasimibe and CP-113818 enhanced production of IFN-γ, TNF-α, and GzmB in human CD8$^+$ T cells as previously suggested[18] (Figure 2A–C), SCD1 inhibitors did not alter the expression of ACAT1 gene or protein (Figure S3A,B). In addition, ACAT1 inhibitors, as well as SCD1 inhibitors, enhanced IFN-γ production by both naïve and memory CD8$^+$ T cells and expression of 4-1BB and TIGIT on CD8$^+$ T cells, although T cell proliferation was not affected (Figure S4A–C).

We then evaluated the effect of SCD1 inhibitors on cholesterol esterification regulated by ACAT1 in CD8$^+$ T cells, and found that SCD1 inhibitors decreased the ratio of esterified cholesterol and cholesterol (Figure 2D left panel). Detailed analysis showed that SCD1 inhibition significantly reduced intracellular esterified cholesterol with little effect on total intracellular cholesterol levels, and this effect on esterified cholesterol by SCD1 inhibitors was canceled by the addition of oleic acid (Figure 2D right panel), suggesting possible involvement of esterified cholesterol in T cell suppression by SCD1. These results indicated that SCD1–ACAT1 axis was important in the T cell suppression by SCD1.

3.3 | Esterified cholesterol suppresses the effector function of human CD8$^+$ T cells

We then evaluated the direct effects of esterified cholesterol, ChO, on CD8$^+$ T cells. Addition of ChO, which had no toxic effect on T cells (Figure S5A), was found to cancel the enhancement of effector functions such as IFN-γ, TNF-α, or GzmB by CD8$^+$ T cells treated in vitro with the SCD1 inhibitors (Figure 2E) and ACAT1 inhibitors (Figure 2A–C), suggesting that the increase of the intracellular esterified cholesterol is one of the important mechanisms for the suppression of effector functions of CD8$^+$ T cells through SCD1-ACAT1 axis. On the other hand, the effect of SCD1 inhibitors in enhancing T cell proliferation was not canceled by the addition of oleic acid and ChO (Figure 1D and Figure S5A), suggesting that the SCD1-ACAT1 axis is not involved in suppressing cell proliferation of CD8$^+$ T cells.

We previously reported that SCD1 inhibitors enhance cell proliferation and chemokine production by mouse CD8$^+$ T cells possibly via reduction of ER stress.[12] Thus, we evaluated the relationship between ER stress response and the suggested ACAT1-dependent immunosuppression. SCD1 inhibitors enhanced cell proliferation of human CD8$^+$ T cells and reduced the expression of sXBP1, ER stress-marker molecules (Figure S5B). However, the addition of esterified cholesterol did not change the ER stress response (Figure S5B) and cell proliferation of CD8$^+$ T cells treated with SCD1 inhibitors (Figure S4C), even though it reversed the enhanced IFN-γ production by T cells treated with SCD1 inhibitors (Figure 2E). These results indicated that esterified cholesterol generated by ACAT1 is one of the responsible molecules for the SCD1-induced suppression of effector functions of CD8$^+$ T cells including the production of IFN-γ, TNF-α, and GzmB, whereas cell proliferation appeared to be regulated by another mechanism such as ER stress.

3.4 | Systemic administration of SCD1 inhibitors enhances the antitumor activity of tumor-infiltrating CD8$^+$ T cells through reduction of immunosuppressive esterified cholesterol

We next evaluated whether the SCD1-ACAT1 axis is also operative in vivo using the MCA205-bearing mouse model (Figure S6). Systemic administration of an SCD1 inhibitor significantly reduced

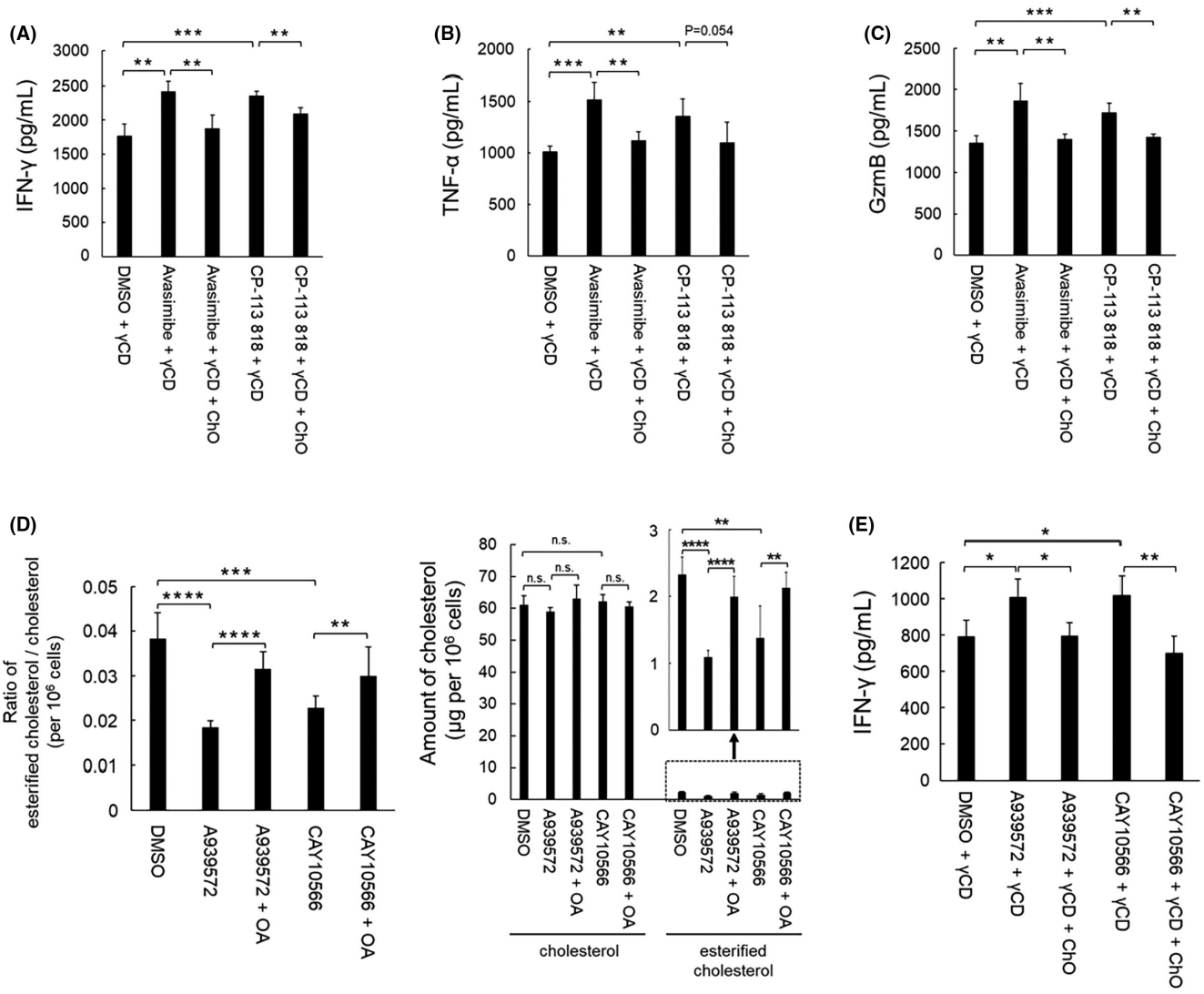

FIGURE 2 Inhibition of SCD1 enhances the effector function of human CD8$^+$ T cells via suppression of esterified cholesterol synthesis by ACAT1. CD8$^+$ T cells were isolated from human PBMCs using MACS and activated by the anti-CD3 mAb, anti-CD28 mAb, and IL-2. (A–C) CD8$^+$ T cells were cultured in AIM-V medium with DMSO + γCD, 1 μM ACAT1 inhibitors (Avasimibe and CP113818) + γCD, or 1 μM ACAT1 inhibitors + γCD + cholesteryl oleate (ChO). IFN-γ (A), TNF-α (B), and GzmB (C) levels in culture supernatants were measured by ELISA. (D) CD8$^+$ T cells were treated with DMSO, SCD1 inhibitors (A939572 and CAY10566), or SCD1 inhibitors + oleic acid (OA). The ratio of esterified cholesterol/cholesterol (left panel) and the amounts of cholesterol and esterified cholesterol (right panel) are shown. (E) CD8$^+$ T cells were cultured in AIM-V medium with DMSO + γ-cyclodextrin (γCD), SCD1 inhibitors (A939572 and CAY10566) + γCD, or SCD1 inhibitors + γCD + cholesteryl oleate (ChO). IFN-γ levels in culture supernatants were measured by ELISA. Data are expressed as means ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n.s., not significant.

oleic acid levels in both tumor tissues and tumor-infiltrating CD8$^+$ T cells (CD8$^+$ TILs; Figure 3A), and reduced esterified cholesterol levels within CD8$^+$ TILs without affecting levels of cholesterol (Figure 3B). IFN-γ production by CD8$^+$ TILs of the SCD1 inhibitor-treated mice was enhanced after ex vivo TIL culture (Figure 3C). In addition, systemic administration of ACAT1 inhibitors also enhanced IFN-γ production by CD8$^+$ TILs in the treated mice (Figure 3D). These results indicated that SCD1 inhibitors also enhanced the antitumor activity in vivo through a decrease in esterified cholesterol in CD8$^+$ TILs.

3.5 | SCD1 inhibitor enhances the antitumor effects of combination cancer immunotherapy

T cell-derived IFN-γ amplifies the T cell response in immunotherapies such as anti-PD-1 antibody therapy.[30,31] Combination of either SCD1 inhibitors or ACAT1 inhibitors with anti-PD-1 mAbs showed synergistic antitumor effects (Figure 3D,E), indicating that interventions on SCD1-ACAT1 axis in CD8$^+$ T cells may be useful for the development of combination immunotherapy.

We also examined whether SCD1 inhibitors enhance the antitumor function of CAR-T cells. Similar to the antitumor CD8$^+$ T cells, in vitro treatment with SCD1 inhibitor significantly reduced intracellular levels of oleate and esterified cholesterol, but not cholesterol, in glypican-1 (GPC1)-specific murine CAR-T (GPC1-mCAR-T) that we have previously developed[23] (Figure 4A,B). The SCD1 inhibitor-treated GPC1-mCAR-T showed enhanced IFN-γ production and tumor cytolysis, when co-cultured with MCA205 tumor cells stably expressed mGPC1 (MCA205-mGPC1; Figure 4C,D). SCD1 inhibitors also enhanced the IFN-γ production and tumor lysis of GPC3-specific human CAR-T (GPC3-hCAR-T) against human cervical cancer cell line, HeLa, transduced with hGPC3 similar to mouse CAR-T cells (Figure S7A,B). Because SCD1 inhibitors enhanced the effector functions of CAR-T cells in vitro, we evaluated the combined effects of GPC1-mCAR-T cells and SCD1 inhibitors using the MCA205-mGPC1-bearing mouse model, and the combination therapy with SCD1 inhibitor and GPC1-mCAR-T cells exhibited stronger

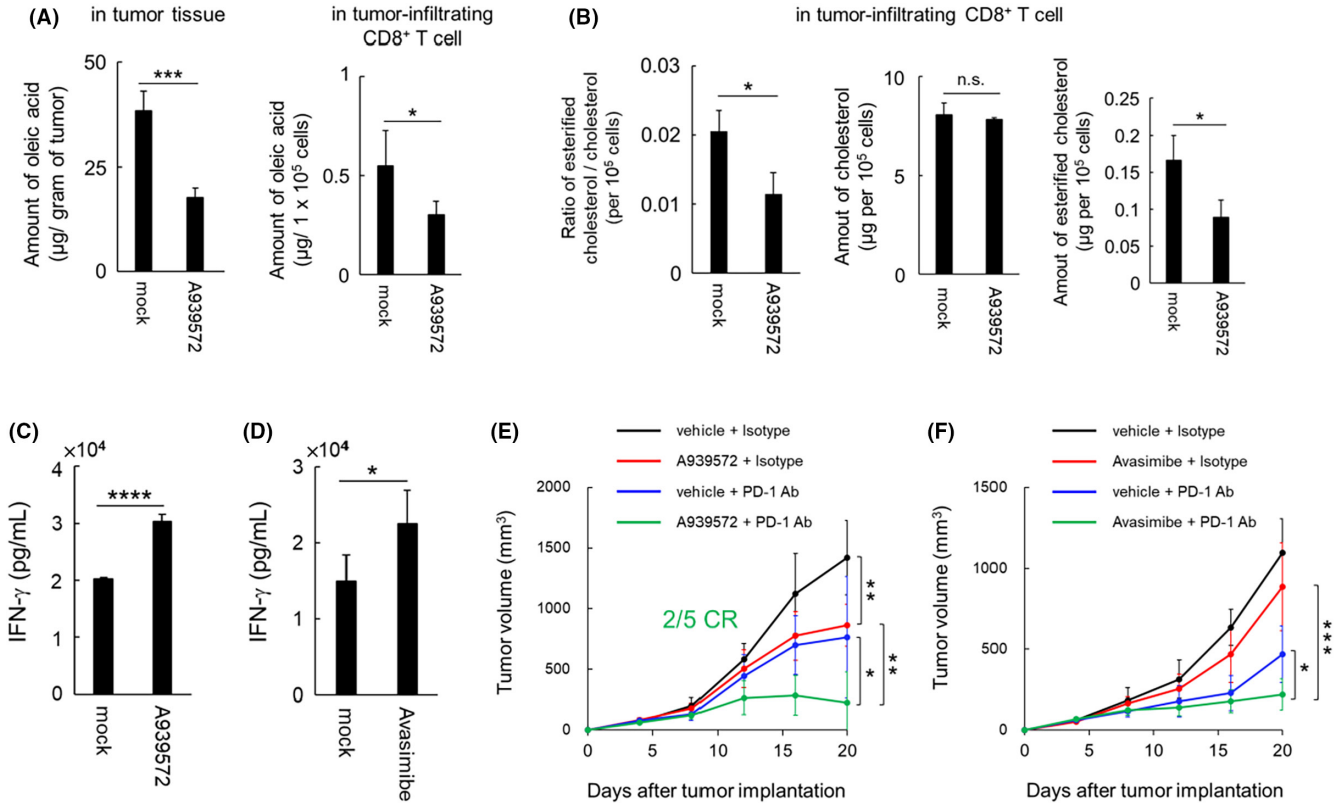

FIGURE 3 Systemic administration of SCD1 inhibitor enhances IFN-γ production by CD8$^+$ T cells in vivo. (A–D) C57BL/6 mice bearing MCA205 tumors were treated with an SCD1 inhibitor (A939572; 10 mg/kg), an ACAT1 inhibitor (Avasimibe; 15 mg/kg) or the vehicle (mock). Tumor-infiltrating CD8$^+$ T cells (CD8$^+$ TILs) were sorted using MACS. (A) Oleic acid levels in tumor tissues and CD8$^+$ TILs. (B) The ratio of esterified cholesterol/cholesterol and the amount of cholesterol and esterified cholesterol in CD8$^+$ TILs are shown. (C, D) CD8$^+$ TILs were cultured ex vivo after SCD1 inhibitor (C) or ACAT1 inhibitor (D) treatment. The next day, IFN-γ levels in culture supernatants were measured by ELISA. Data are expressed as means ± SD ($n = 5$). (E) Mice bearing MCA205 tumors were treated with A939572 or the vehicle and anti-PD-1 antibody (200 μg/mouse) or isotype-matched antibody. (F) Mice bearing MCA205 tumors were treated with an ACAT1 inhibitor (Avasimibe; 15 mg/kg) or the vehicle and anti-PD-1 antibody (200 μg/mouse) or isotype-matched antibody. Tumor growth curves for average tumor volumes are shown (means ± SD; $n = 5$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n.s., not significant.

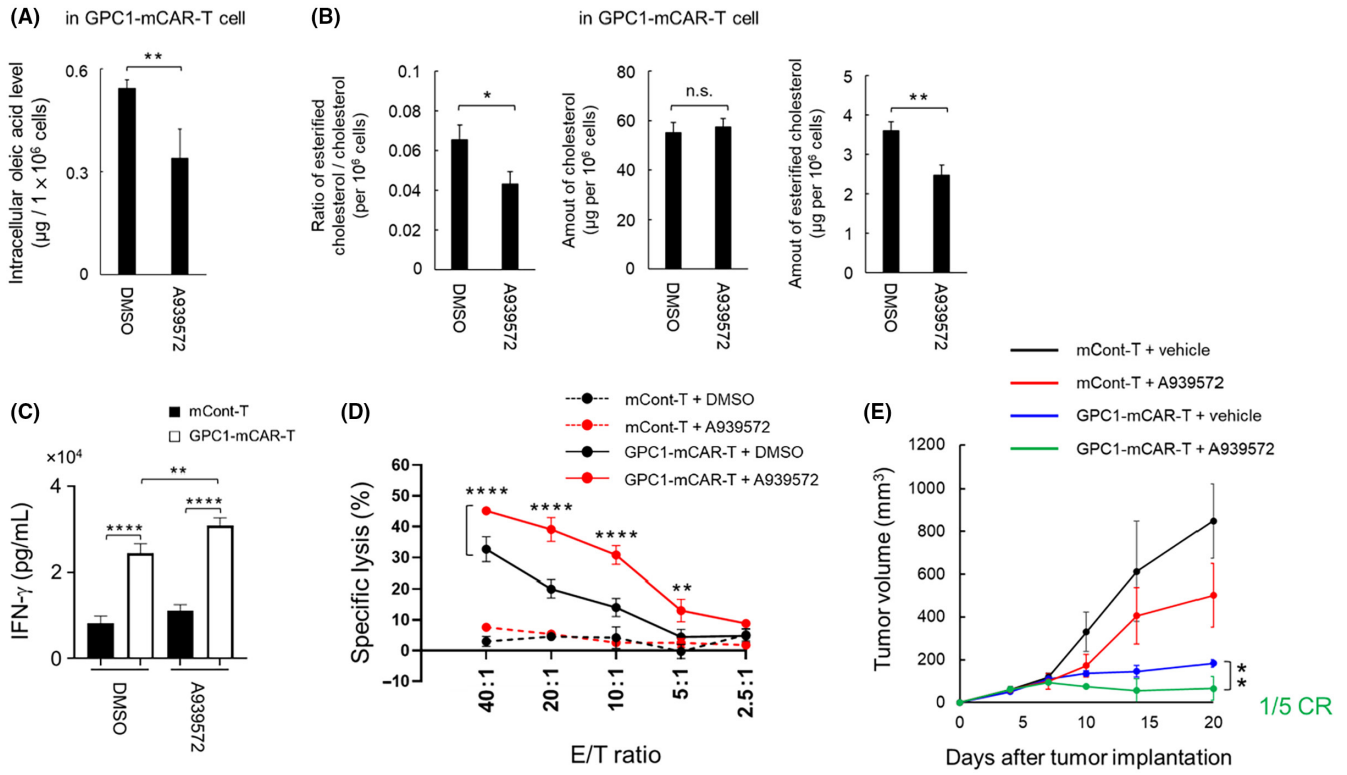

FIGURE 4 SCD1 is a potential target for enhancing the antitumor effects of CAR-T cell therapy. GPC1-specific murine CAR (GPC1-mCAR)-T cells and murine control T (mCont-T) cells were generated as described in Section 2 and then treated with DMSO or A939572. (A) Intracellular oleic acid levels in GPC1-mCAR-T cells. (B) The ratio of esterified cholesterol/cholesterol and the amount of cholesterol and esterified cholesterol in GPC1-mCAR-T cells are shown. (C, D) GPC1-mCAR-T cells or mCont-T cells and mGPC1-overexpressing MCA205 tumor cells (MCA205-mGPC1) were co-cultured in vitro. (C) IFN-γ secretion was evaluated by ELISA. (D) Cytotoxic activity was evaluated using a standard Cr^{51}-releasing assay. Data are expressed as means ± SD ($n = 3$). (E) Mice bearing MCA205-mGPC1 were treated with 5×10^6 cells of GPC1-mCAR-T cells or mCont-T cells on day 2 and SCD1 inhibitor was administered by oral gavage twice daily for 16 days starting on day 4. Tumor growth curves for average tumor volumes are shown (means ± SD; $n = 5$). $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$. E/T ratio, effector cell (GPC1-mCAR-T cells)/target cell (MCA205-mGPC1 cell) ratio.

antitumor activity than those of GPC1-mCAR-T cells alone or SCD1 inhibitor alone (Figure 4E). These results strongly suggest that the SCD1-ACAT1 axis is an important target to improve the therapeutic efficacy of combination cancer immunotherapy with anti-PD-1 antibodies or CAR-T cell therapy.

4 | DISCUSSION

Understanding the mechanisms involved in non-T cell-inflamed immune resistance is important to improve the efficacy of cancer immunotherapy such as immune checkpoint inhibitors. Recent attention has focused on immune metabolism including lipid metabolism in the tumor microenvironment as a target for interventions to enhance antitumor immune responses.[32] We previously demonstrated that fatty acid desaturase SCD1 has an immunosuppressive activity in cancer cells and T cells, and inhibition of its activities enhances the antitumor activity of anti-PD-1 antibodies through increased production of DC-recurring chemokines such as CCL4 by cancer cells and T cells in mouse tumor models. In addition, we reported the correlations between SCD1-related serum-free fatty acid levels and the response to anti-PD-1 antibodies in non–small-cell lung cancer patients.[12] In this study, we demonstrated a novel mechanism of SCD1 inhibition to directly enhance effector functions of human and mouse antitumor $CD8^+$ T cells by inhibiting SCD1-oleic acid-ACAT1-esterified cholesterol pathway, and proposed that the interventions on SCD1-ACAT1 axis may be useful for development of combination cancer immunotherapy with immune checkpoint inhibitors or CAR-T cell therapy (Figure 5).

In this study, we found that intracellular oleic acid and subsequent esterified cholesterol, but not palmitoleic acid and cholesterol, that are regulated by the SCD1-ACAT1 axis are important for the effector functions of antitumor $CD8^+$ T cells, although the mechanism of immunosuppression by esterified cholesterol needs further investigation. Recent studies have demonstrated that various fatty acids are important regulators of T cell functions.[33] Ma et al.[34] indicated that arachidonic acid, a polyunsaturated fatty acid, decreases IFN-γ production by $CD8^+$ T cells via increased lipid peroxidation and ferroptosis. Nava Lauson et al.[35] reported that linoleic acid is a major positive regulator of $CD8^+$ T cell effector functions via improving

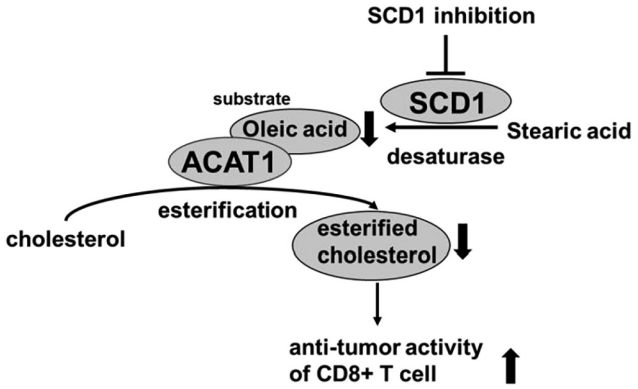

FIGURE 5 Schematic of the study. Inhibition of SCD1 enhances the antitumor activity of $CD8^+$ T cells by reducing the intracellular oleic acid level, ACAT1-dependent cholesterol esterification reaction, and esterified cholesterol level.

mitochondrial fitness and preventing exhaustion. Recent reports also indicated that intracellular cholesterol also plays a major role in the regulation of T cell antitumor activity. Yang et al.[18] reported that elevated cholesterol levels in the plasma membrane of $CD8^+$ T cells promote TCR clustering and signaling, resulting in markedly enhanced production of cytokines and cytotoxic granules. Ma et al.[36] reported that cholesterol accumulation in $CD8^+$ T cells promotes the expression of immune checkpoint molecules by increasing ER stress, leading to $CD8^+$ T cell exhaustion. We have not understood the entire picture of lipid metabolism in tumor immune microenvironments including cross-talks of fatty acid and cholesterol metabolism. We demonstrated that the SCD1–ACAT1 fatty acid-cholesterol axis was one of the key regulators of the effector function of antitumor $CD8^+$ T cells. The adequate levels of fatty acids and cholesterol and their metabolites appear to be important for antitumor T cell functions, and it warrants further investigation.

To date, various metabolic modulators, such as bezafibrate,[37,38] a PPAR agonist, and 6-diazo-5-oxo-l-norleucine,[39] a glutamine metabolic antagonist, have been reported to improve the immunosuppressive cancer microenvironment and enhance the efficacy of anti-PD-1 antibody therapy. In this study, we also showed the potential of combination cancer immunotherapy targeting the SCD1–ACAT1 axis along with anti-PD-1 antibody or CAR-T therapy, because those inhibitors enhanced effector functions including the production of IFN-γ, an important antitumor cytokine. IFN-γ signaling dysfunction in cancer cells was reported to be the major mechanism of acquired resistance to anti-PD-1 antibody therapy.[30] Our results demonstrated that targeting the SCD1–ACAT1 lipid metabolism pathway may also be an attractive therapeutic strategy to improve the therapeutic efficacy of cancer immunotherapy.

In summary, we found that SCD1 suppressed the effector functions of antitumor $CD8^+$ T cells through an increase in immunosuppressive oleic acid-related esterified cholesterol generated by ACAT1, indicating the potential use of SCD1 inhibitors or ACAT1 inhibitors for combination cancer immunotherapies with immune checkpoint inhibitor or CAR-T cell therapy. Targeting the SCD1–ACAT1 axis is an attractive cancer therapy, because SCD1 or ACAT1 inhibition was also reported to work on cancer cells.

AUTHOR CONTRIBUTIONS

Toshihiro Sugi: Conceptualization; formal analysis; methodology; writing – original draft; writing – review and editing. **Yuki Katoh:** Conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; visualization; writing – original draft; writing – review and editing. **Toshikatsu Ikeda:** Formal analysis. **Daichi Seta:** Formal analysis. **Takashi Iwata:** Investigation. **Hiroshi Nishio:** Investigation. **Masaki Sugawara:** Formal analysis. **Daiki Kato:** Formal analysis. **Kanoko Katoh:** Formal analysis. **Kei Kawana:** Investigation. **Tomonori Yaguchi:** Conceptualization; methodology; project administration; resources; writing – original draft; writing – review and editing. **Yutaka Kawakami:** Conceptualization; funding acquisition; methodology; project administration; resources; writing – original draft; writing – review and editing. **Shuichi Hirai:** Formal analysis; investigation.

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CONFLICT OF INTEREST STATEMENT

Yutaka Kawakami is an editorial board member of Cancer Science and other authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or as online Supporting Information.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The use of human specimens was approved by the Keio University School of Medicine Ethics Committee (approval number: 20130122).

Informed Consent: All participants provided written informed consent before participation in the study.

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

Animal Studies: All animal studies were reviewed and approved by the Nihon University Institutional Animal Care and Use Committee (approval number: AP20MED031-3) and the Keio University Institutional Animal Care and Use Committee (No. 4062). Recombinant DNA experiments were reviewed and approved by the Genetic Modification Safety Committee, Keio University School of Medicine (No. 25-014-13).

ORCID

Yuki Katoh https://orcid.org/0000-0002-7467-3377

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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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