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Fatty acid uptake mediated by FABP4 promotes the formation of CD8⁺T cell senescence through lipid peroxidation in the adipocyte-rich microenvironment of Ovarian Cancer

Chunyan Yu^{1,11}, Xin Li^{2,11}, Xiaolong Qian^{3,11}, Haoke Zhang^{1,11}, Xueying Li¹, Bo Wang¹, Mantong Li⁴, Zixuan Liu⁵, Wei Du¹, Siqi Chen¹, Yuqing Ouyang¹, Xiaofan Feng¹, Tianhui He⁶, Zihe Liu¹, Haixia Wu⁷, Xiaoyan Zheng⁸, Junru Liu⁹, Hong Zhang¹, Yuanming Song³, Chenying Liu³, Jiazhen Li³, Hongyan Guo⁶, Shiwen Xu¹⁰, Xiaojing Guo³ and Weimin Deng¹

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T cell senescence significantly impairs the efficacy of immune checkpoint blockade (ICB) therapy in cancer. Metabolic reprogramming is a crucial factor in T cell senescence in tumor microenvironment (TME). Ovarian cancer (OvCa) patients derive limited benefit from ICB treatment, probably related to T cell senescence. OvCa cells metastasize to the abdominal cavity rich in omental fat and raise ascites, forming a unique TME, adipocyte-rich TME. In this study, we investigated the effects of adipocyte-rich TME on T cell senescence. Using the single-cell RNA sequencing of OvCa and clinical samples, we found that adipocyte-rich TME is strongly associated with the formation of senescence CD8⁺T (CD8⁺Tsen) cells. Mechanistically, adipocyte-derived factors (MATES) and oleic acid (OA)-the predominant fatty acid in OvCa ascites-promoted tumor-induced CD8⁺Tsen formation by enhancing fatty acid (FA) uptake via FABP4, triggering lipid peroxidation rather than energy production. Inhibition of *FABP4* (using the inhibitor BMS309403 or siRNA knockdown) blocked CD8⁺Tsen cell formation, reduced lipid peroxidation, restored CD8⁺T cell effector function, and suppressed immunosuppressive cytokines. Moreover, using an OvCa mouse model, we found that in OvCa mice BMS309403 treatment partially diminished CD8⁺Tsen formation by reducing FA uptake, and improved anti-tumor immunity, and prolonged the survival time of OvCa mice when combined with chemotherapy. Our work suggests *FABP4*-mediated FA metabolism as a therapeutic target to counteract T cell senescence in adipocyte-rich TME, providing a novel immunotherapeutic strategy for OvCa.

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INTRODUCTION

In the past two decades, cancer immunotherapies primarily based on T cells have developed rapidly, among which immune checkpoint blockade (ICB) therapy is the most representative therapy [1]. However, the overall success rate of ICB therapy remains limited [2]. T-cell dysfunctions, including exhaustion and senescence, are crucial factors that hinder the success of cancer immunotherapies [3] and essential targets to improve anticancer immunity [4]. CD8⁺T cell senescence, caused by significant inhibition of the tumor microenvironment (TME), is an important strategy for malignant tumors to evade immune surveillance and one of the possible reasons for the poor efficacy of ICB [5].

Senescent T (Tsen) cells not only display the common features of senescent cells including upregulation of age-related

β-galactosidase (SA-β-gal), lipofuscin, cell cycle-related factors (p16, p21, p53, etc.) [6], and demonstrate senescence-associated secretory phenotype (SASP) with up-regulated pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-8, etc. [7–9], but also exhibit immunological characteristics that are different from other dysfunctional T cells like exhausted T (Tex) cells, including downregulation of CD27 and CD28 [8, 10], etc. These features enable Tsen cells to cause senescence-related diseases, including various malignancies [5]. CD8⁺CD28[−] T cells have been defined as Tsen cells in multiple human cancers and aging [10] and exhibit immunosuppressive effects [3, 11]. The failure of ICB therapy in glioblastoma patients is mainly due to CD8⁺CD28[−] T cells, rather than Tex cells since the former lacks the main ICB targets [12]. It is widely believed that T cell senescence is irreversible [3]. Hence,

¹Tianjin Institute of Immunology, Department of Immunology, Tianjin Key Laboratory of Cellular and Molecular Immunology, Key Laboratory of Diseases and Microenvironment of Ministry of Education of China, Tianjin Medical University, Tianjin, China. ²Department of Gerontology, The Second Hospital of Tianjin Medical University, Tianjin, China. ³Department of Breast Pathology and Lab, Tianjin Medical University Cancer Institute & Hospital, National Clinical Research Center for Cancer, Key Laboratory of Breast Cancer Prevention and Therapy of Ministry of Education of China, Tianjin Medical University, Tianjin's Clinical Research Center for Cancer, Tianjin, China. ⁴Department of Apheresis Platelets, Tianjin Blood Center, Tianjin, China. ⁵Department of Medical Technology, Shijiazhuang Medical College, Shijiazhuang, Hebei Province, China. ⁶Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing, China. ⁷Department of Pathology, Tianjin Central Hospital of Gynecology Obstetrics, Tianjin, China. ⁸Department of Laboratory, Shanxi Eye Hospital, Taiyuan, Shangxi Province, China. ⁹Department of blood transfusion, Qilu Hospital of Shandong University Dezhou Hospital, Dezhou, Shandong Province, China. ¹⁰Tianjin Niusai Biotechnology Co., Ltd, Tianjin, China. ¹¹These authors contributed equally: Chunyan Yu, Xin Li, Xiaolong Qian, Haoke Zhang.

✉email: ntcell@163.com; guoxiaojing@tjmuch.com; dengweimin@tmu.edu.cn

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preventing the formation of Tsen cells becomes a promising anticancer immunotherapy strategy.

Recently, the effect of TME on the metabolic fitness of T cells has attracted much attention, and the metabolic function of T cells is regarded as a target to enhance anticancer immunity and improve immunotherapy efficacy [13]. In TME, the cancer cells competitively intake nutrients including glucose and glutamine to maintain their rapid growth and proliferation [14], which results in nutrient stress for T cells. Preclinical and clinical studies reported that T cells catabolize lipids to sustain energy supply for functions through fatty acid (FA) oxidation (FAO) [15] due to nutrient stress in TME. However, recent studies have revealed that lipid metabolites in the TME, particularly oxidized lipids, can inhibit T cell function. For example, Shihao Xu et al. reported that the uptake of oxidized lipids by the FA transport molecule CD36 suppressed the tumor-killing function of cytotoxic T lymphocytes (CTLs) in melanoma and colorectal adenocarcinoma [16]. Yuma Saimoto et al. reported that oxidized lipids increase T cells susceptibility to oxidative stress by downregulating the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [17]. Mahdi Ghatreh-Samani et al. reported that oxidative stress can induce T cell senescence [18]. Currently, metabolic reprogramming is recognized as a key factor in T cell senescence within the TME. However, studies addressing the intervention of lipid metabolism to affect T cell senescence from the perspective of the TME have not been reported.

Ovarian cancer (OvCa) is a fatal female gynecological malignancy [19]. Epithelial ovarian cancer (EOC) is the most common histological type of OvCa. High-grade serous ovarian cancer (HGSOC) is the major subtype of EOC [20] and more than 70% of HGSOC patients have been in the advanced stage (International Federation of Gynecology and Obstetrics (FIGO) stage III/IV) when diagnosed [20]. The first-line treatment for HGSOC is tumor reduction and chemotherapy based on platinum and paclitaxel. However, many patients relapse after the treatments, and the 5-year survival rate of patients is only 25%–30% [21]. The efficacy of ICB therapy for HGSOC patients is poor [22]. EOC cells extensively metastasize to the abdominal cavity that is rich in omental fat and finally raise ascites [20], forming a unique TME, adipocyte-rich TME [23]. Tsen cells are closely related to OvCa progression [7]. Patients with more Tsen cells in ascites exhibit a shorter progression-free survival [24]. Ascites mainly contain FAs, the primary extracellular lipids, and adipokines [25, 26]. Therefore, the contributions of FA metabolism in T cell senescence in OvCa require further in-depth studies.

In this study, we systematically investigated the impact and mechanisms of adipocyte-rich TME on the formation of CD8⁺Tsen using bioinformatics data, clinical specimens, EOC cell model and mouse model. Thereby, we furthermore used tumor-bearing mouse model to evaluate the feasibility of decreasing CD8⁺Tsen formation by inhibiting FA uptake, and enhancing anti-cancer immunity in combination with chemotherapy. This research provides new insights for targeting dysfunctional T cells in anti-cancer immunotherapy from the perspective of FA metabolism.

MATERIALS AND METHODS

Cell lines and cell culture

The mouse ovarian epithelial papillary serous adenocarcinoma cell line ID8 (C57BL/6 gene background) was a gift from Professor Luyuan Li in 2018 (Nankai University, Tianjin, China). ID8 cells were cultured in DMEM, and T cells were cultured in RPMI 1640 medium (Jiangsu Kaiji Biotechnology Co. LTD.), both containing 80 U/ml of penicillin and 80 µg/ml of streptomycin (PS), supplemented with 10% (v/v) FBS (Gibco). The ID8 cells were routinely tested for mycoplasma and were passaged less than 10 times. The cells were usually passaged 1–2 times and then used in subsequent experiments.

Clinical tissue samples

For immunohistochemistry (IHC), ovarian cancer tissue samples from 20 HGSOC patients were collected at the Tianjin Central Obstetrics and Gynecology Hospital from 2022 to 2023. These tissue samples were confirmed by histopathological examination, and before use, informed consent of the patient was obtained as authorized with the Ethics Committee of Tianjin Central Obstetrics and Gynecology Hospital.

Preparation of mouse adipocyte tissue extracts (MATES)

MATES was prepared according to the procedures isolating extracts from human adipose tissue [27]. Briefly, the adipose tissue of the pelvic and lower abdomen of mice was obtained aseptically, washed 2–3 times, and centrifuged at 1200 × g for 3 min to remove tissue debris. The red blood cells were removed with red blood cell lysate (Beijing Tiangen Biochemical Technology Co., LTD.; Cat# RT122) according to the manufacturer's instructions. Then the adipose tissue was mechanically emulsified and centrifuged at 1200 × g for 5 min and the supernatant was collected. MATES was obtained after filtering the supernatant through a 0.22 µm filter. MATES was frozen in a –80 °C refrigerator at a concentration of 0.2 g/ml and was diluted with regular medium containing 20% FBS at a ratio of 1:1 for in vitro studies.

Magnetic cell sorting (MACS)

The spleen (SP) of C57BL/6 mice was obtained aseptically to prepare a single-cell suspension, and the red blood cells were removed with red blood cell lysate (Beijing Tiangen Biochemical Technology Co., LTD.; Cat# RT122) according to the manufacturer's instructions. Peritoneal lavage fluid was collected aseptically to obtain ascites and the red blood cells were removed with red blood cell lysate. The cells were resuspended in a culture dish and cultured at 37 °C for 2 h to allow cancer cells to adhere to the wall, and the lymphocytes in the supernatant were collected. Single-cell suspension was prepared according to the manufacturer's instructions. ImunoSep mouse CD8⁺T cell enrichment kit (Precision BioMedicals; Cat# 720805) was applied to obtain CD8⁺T cells.

In vitro treatment of T cells

To establish the Tsen-cell-induction model, 1 × 10⁶/ml splenic CD8⁺T cells of tumor-bearing mice (tumor-bearing for 2 w) were seeded in 12-well plate (6 × 10⁶/well) and cocultured with ID8 cells (at a ratio of 8:1) and MATES at different concentrations for 48 h. In the mechanistic study, 1 × 10⁶/ml splenic CD8⁺T cells from tumor-bearing mice were pretreated with 25 µM BMS309403 (BMS, MedChemExpress; Cat# HY-101903), 10 µM MHY1485 (MHY, MedChemExpress; Cat# HY-B0795), or 10 µM rapamycin (Rapa, MedChemExpress; Cat# HY-10219) for 2 h, respectively. Then the cells were washed twice with PBS, seeded in 12-well plates (5 × 10⁵/well), cocultured with ID8 cells, and treated with 150 µM oleic acid (OA) for 48 h. T cells were then collected and analyzed by flow cytometry.

FABP4 knockdown and overexpression

siRNAs targeting mouse FABP4 gene (siFABP4) and nonsense siRNA (negative control, siNC) were designed and synthesized by RiboBio (Guangzhou, China). The sequence of siFABP4-1 is: GGGCTTTGCCACAAG-GAAA and the sequence of siFABP4-2 is: GGTGAATGTGTTATGAAA. The mouse FABP4 over-expression plasmid (pCMV-Fabp4(mouse)-3×FLAG-Neo) and nonsense plasmid (pCMV-3×FLAG-MCS-GST-SV40-Neo) were designed and synthesized by MiaoLing Plasmid Platform (WuHan, China). Mouse CD8⁺T cells were collected and transfected with siRNA/plasmid oligonucleotides (200 pmol/100 µl) for 30 min using ProteanFect Max Mouse Primary T Cell Transfection Kit (Nanoportal Biotech; Cat#: PT03) according to the manufacturer's instructions. After 30 min, the transfection medium was replaced with a regular medium and then incubated at 37 °C in 5% CO₂. CD8⁺T cells were collected 24 h after transfection for subsequent studies.

Trp53 Knockdown

DNA oligos encoding a sgRNA specific for Trp53 were ligated into the lentiCRISPRv2 vector. HEK-293T cells were transfected with packaging and target plasmids using Lipofectamine 3000 (Thermo Fisher Scientific). Lentivirus-containing supernatant was collected at 48 and 72 h and used to infect ID8-luc cells (maintained in laboratory). After transduction, use puromycin to select for cells with lentiCRISPRv2.

Flow cytometry and Fluorescence-activated cell sorting (FACS)

The prepared single cell suspension (1×10^6 /ml) of CD8⁺T cells was resuspended in Zombie NIR live/dead stain (Biolegend; Cat# 423105) and incubated for 15 to 30 min at room temperature. Cells were washed twice, then resuspended in 100 μ l FACS buffer (2% FBS, 1 mM EDTA in PBS) and incubated with fluorescence-labeled Abs. For the detection of granzyme B (GZMB), Fas ligand (FasL), interferon- γ (IFN- γ), and IL-6, CD8⁺T cells were processed with Cell Activation Cocktail (Biolegend; Cat# 423303) for 6 h and then incubated with corresponding Abs. The fluorescent-labeled Abs included anti-mouse Percp-Cyanine5.5-CD45 (0.25 μ l, Invitrogen; Cat# 45-0451-82, RRID: AB_1107002), anti-mouse PE-CD3 (0.25 μ l, Biolegend; Cat# 100206, RRID: AB_312663), anti-mouse PE/Cyanine7-CD3 (0.2 μ l, Biolegend; Cat# 100220, RRID: AB_1732057), anti-mouse APC-CD8 (0.25 μ l, Biolegend; Cat# 100782, RRID: AB_2819775), anti-mouse PE-CD326 (0.25 μ l, Biolegend; Cat# 118205, RRID: AB_1134176), FITC-Bodipy-FL-C16 (0.2 μ l, Invitrogen; Cat# D3821, RRID: AB_324734), 581/589-Bodipy-C11 (0.2 μ l, Beyotime; Cat# S00435), anti-mouse FITC-IFN- γ (1 μ l, Invitrogen; Cat# 11-7311-81, RRID: AB_465411), anti-mouse PE-GZMB (0.625 μ l, Invitrogen; Cat# 12-8898-80, RRID: AB_10853811), anti-mouse APC-FasL (1 μ l, Biolegend; Cat# 106609, RRID: AB_2813951), anti-mouse PE-IL-6 (0.625 μ l, Biolegend; Cat# 504503, RRID: AB_315337), anti-mouse APC-IL-10 (0.625 μ l, Invitrogen; Cat# 17-7101-81, RRID: AB_469501), PE-Ki67 (1 μ l, Biolegend; Cat# 151209, RRID: AB_2716014), FITC-FASL (1 μ l, Invitrogen; Cat# 11-5911-82, RRID: AB_11150968), APC-CD4 (1 μ l, Biolegend; Cat# 100411, RRID: AB_312696), FITC-IL2 (1 μ l, Invitrogen; Cat# 11-7021-82, RRID: AB_465382) and anti-mouse/human FITC-Ki67 (1 μ l, Biolegend; Cat# 151211, RRID: AB_2814054). The cellular senescence detection Kit-SPiDER- β -gal (Dojindo; Cat# SG03) was used to determine Tsen cells. Annexin V-FITC/PI Apoptosis Detection Kit (Jiangsu Kaiji Biotech; Cat# KGA1102) was applied to determine the apoptosis of ID8 cells. The procedures for the staining of intracellular factors were as follows: The cell surface antigens including CD45, CD3, CD8, FasL and Ki67 were stained first, then 4% paraformaldehyde was added for fixation. Intracellular Staining Permeabilization Wash Buffer (10X) (Biolegend; Cat# 421002) was used to permeabilize cells after fixation, and then the intracellular factors including IFN- γ , GZMB, IL-6 and IL-10 were stained with corresponding Abs. The cells were detected using a flow cytometer (BD Bioscience) and analyzed using FlowJo _V10 software.

RNA sequencing (RNA-seq)

Splenic CD8⁺T cells were seeded in a 75 cm² flask (1×10^6), and cocultured with ID8 and MATES for 48 h. Then, suspended CD8⁺T cells were isolated using a mouse lymphocyte separator (Tianjin Haoyang Huake Biotechnology Co., Ltd.; Cat# LTS1092). 1 ml TRIzol (Tiangen Biotech, CO., Ltd.; Cat# DP424) reagent was added to lyse the cells, and the samples were sent to Tiangen Biotech, CO., Ltd. for RNA sequencing and analysis. According to the manufacturer's instructions, RNA with Poly-A structure was enriched from total RNA using the TIANSeq mRNA capture kit (TIANGEN Biotech). Then, the captured RNA was used as the starting sample for library construction with the TIANSeq rapid RNA library construction kit (Illumina Platform, TIANGEN Biotech). The RNA library was then analyzed with the Agilent 2100 Bioanalyzer. After the library test was qualified, PE150 sequencing was performed using the Illumina Platform to obtain 150 bp paired-end sequencing reads. The data can be found in Sequence Read Archive (SRA accession PRJNA1181575).

Lipidomics

Spleen CD8⁺T cells (5×10^5) were cultured in 10 cm petri dishes with ID8 (6×10^5) and OA for 48 h. The supernatant was collected and centrifuged, followed by discarding the supernatant. The cell precipitate was transferred to a cryovials for rapid freezing and sent to AZENTA for lipidomics analysis. According to the company's specifications. In this study, lipid metabolites were detected and then the missing values were filled up by the half of minimum. The final dataset containing the information of compound name, sample name and concentration was imported to SIMCA18.0.1 software (Sartorius Stedim Data Analytics AB, Umea, Sweden) for multivariate analysis. Data was scaled and logarithmic transformed to minimize the impact of both noise and high variance of the variables. After these transformations, PCA (principle component analysis, PCA), an unsupervised analysis that reduces the dimension of the data, was carried out to visualize the distribution and the grouping of the samples. 95% confidence interval in the PCA score plot was used as the threshold to identify potential outliers in the dataset.

Immunohistochemical (IHC) staining

IHC staining of tumors from human HGSOc patients from Tianjin Central Obstetrics and Gynecology Hospital was performed as previously described [28] with primary Ab against human CD8 (rabbit, 1:100, Affinity; Cat# AF5126, RRID: AB_2837612) and secondary Ab (Sheep anti-Rabbit IgG polymer, Beijing Zhongshan Jinqiao Biological Co., LTD.; Cat# PV-6001, RRID: AB_2864333). In brief, after dewaxing and hydrating the specimen, heat-induced antigen repair was performed with citrate buffer (pH = 6.0) and returned to room temperature. The endogenous peroxidase was removed, and goat serum was dropped for blocking at room temperature for 20 min. The specimen was incubated with anti-CD8 Ab at 4 °C overnight, then secondary Ab was added at room temperature, and the color reaction was performed using DAB. Finally, hematoxylin was used to counterstain the nucleus, and images were collected under an optical microscope (ZEISS) for analysis. The number of CD8⁺T cells was counted and averaged in 3 independent areas adjacent to (within 1 mm of the margin zone of the closest adipose tissue) [29] or distant from adipose tissue in each specimen, representing the number of CD8⁺T cells in the specimen.

Sudan Black B (SBB) staining

SBB (MedChemExpress; Cat# HY-D0213) staining was performed to detect the deposition of intracellular lipofuscin [30]. Briefly, the tissue specimen was dewaxed with xylene and hydrated with a gradient of concentration of ethanol (from anhydrous ethanol, 95%, 80%, and finally to 70%), immersed in freshly prepared SBB solution for 30 min, then quickly transferred to 50% ethanol, washing twice to avoid deposition of SBB stains on the tissue, and finally redyed with an appropriate amount of 0.1% nuclear solid red solution (Jiangsu Kaiji Biotech. Co. LTD.; Cat# KGA232) for 1 min, sealed with neutral gum for storage. Optical microscopy (ZEISS) was applied to capture 3 visual fields adjacent to or away from adipose tissue in HGSOc patient specimens. For the quantification of SBB, RGB images were converted into RGB stack format in ImageJ, and the SBB-stained positive (SBB⁺) area in each visual field was displayed by a blue channel and quantified as the percentage of the field area. The 3 values in 3 different areas of each specimen were averaged, respectively, representing the proportion of SBB⁺ cells.

Co-staining of IHC and SBB

The procedure involved initial HC staining to label cell surface CD8 molecules, followed by SBB staining to detect intracellular lipofuscin deposition. Briefly, after deparaffinization and hydration of the specimens, heat-induced antigen retrieval was performed using citrate buffer (pH = 6.0), followed by cooling to room temperature. After blocking endogenous peroxidase activity, the samples were incubated with goat serum at room temperature for 20 min for blocking. The specimens were then incubated with anti-CD8 antibody at 4 °C overnight, followed by the application of a secondary antibody at room temperature and DAB chromogenic development. Subsequently, ethanol dehydration was carried out in a graded concentration series (from 50% to 70%). The samples were immersed in a freshly prepared SBB solution in 70% ethanol for 15 min, quickly transferred to 50% ethanol for two washes to prevent dye precipitation, and finally counterstained with hematoxylin for nuclei [31]. Images were acquired using an optical microscope (Zeiss) for analysis. The ratio of CD8 and lipofuscin double-positive cells to CD8 single-positive cells was statistically calculated in three independent areas adjacent to or distant from adipose tissue in each.

Reverse transcription-polymerase chain reaction (RT-PCR)

Mouse CD8⁺T cells were collected and homogenized for RNA extraction following the manufacturer's instructions: total RNA of 1×10^6 CD8⁺T cells was extracted with TRIzol and the concentration was detected, and TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing Quanshi Gold Bio. Co., LTD.; Cat# AT311-03) were used to extract cDNA and performed RT-PCR using primers (see Table S1). PCR products were analyzed using agarose gel electrophoresis. β -actin was used as the internal reference to calculate the relative mRNA levels.

A-CoA and ATP level detected by ELISA

Mouse CD8⁺T cells were homogenized in cold PBS and centrifuged at $12,000 \times g$ at 4 °C for 10 min to collect the supernatant. The content of A-CoA and ATP was quantified using a mouse A-CoA ELISA Kit

(LunChangShuoBiotech; Cat# ED-21683) and ATP ELISA Kit (LunChangShuoBiotech; Cat# ED-24959). The absorbance was determined at 450 nm (OD450) using a microplate reader (BioTek Instruments, Inc.). A standard curve of mouse A-CoA and ATP was generated to quantify the concentration of A-CoA and ATP.

FAO rate measurement

Mouse CD8⁺T cells were homogenized in cold PBS and centrifuged at 12,000 × *g* at 4 °C for 10 min to collect the supernatant. FAO rate was evaluated with a colorimetric assay kit (Elabscience; Cat# E-BC-K784-M) according to the provider's instruction. After standardizing the protein concentration (BCA Protein Assay Kit, KeyGEN; Cat# KGB2101-250), the absorbance at 450 nm was measured. The amount of enzyme in 1 g protein/min that hydrolyzes the substrate to produce NADH at 37 °C was defined as 1 unit by the following formula: FAO ability (U/gprot) = (ΔA_{450-b}) / $a \div T \times 1,000 / \text{Cpr}$ (ΔA_{450} : OD sample – OD control. T: reaction time, 30 min. Cpr: concentration of protein in sample).

Western Blot (WB) analysis

After harvesting the cells, RIPA (Jiangsu Kaiji Biotech. Co. LTD.; Cat# KGB5204) was used to lyse the cells. Phosphatase inhibitors (Jiangsu Kaiji Biotech. Co. LTD.; Cat# KGB5101-2) and protease inhibitors (Jiangsu Kaiji Biotech. Co. LTD.; Cat# KGB5101-100) were added to inhibit phosphatase and protease activity. The proteins were separated on SDS-PAGE (10% or 12%) according to molecular weight, transferred to a PVDF membrane, incubated at 4 °C overnight with primary Abs, and then incubated with the corresponding secondary Ab. The information on primary Abs is listed in Table S2. Protein bands were scanned with Odyssey[®]CLx. The density of the bands was detected using Odyssey 3.0 software.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 kit (Jiangsu Kaiji Biotech. Co. LTD.; Cat# KGA317) was used to detect the viability of ID8 cells after CD8⁺T cell-killing and the proliferation of CD8⁺T cells in the coculture system. After $1.2 \times 10^3 / 100 \mu\text{l}$ ID8 cells were cocultured with $1 \times 10^4 / 100 \mu\text{l}$ CD8⁺T cells in a 96-well plate for 48 h, the suspended CD8⁺T cells were isolated by washing with PBS twice and transferred into another 96-well plate. Then, CD8⁺T cells and ID8 cells were added with RPMI 1640 medium containing 10% CCK-8 reaction solution and incubated at 37 °C for 1.5 h, respectively. The absorbance was determined at 450 nm (OD450) using a microplate reader (BioTek Instruments, Inc.). The OD450 value of the control was used as the baseline of proliferation and was set as 100%, and the other groups were normalized to the control group.

Animal experiment

All the C57BL/6 mice (17–18 g, 8 w, female) were purchased from Beijing Sibeifu Animal Company. ID8/ID8-luc-P53^{-/-} cells ($3 \times 10^6 / 200 \mu\text{l}/\text{mouse}$) were intraperitoneally injected into C57BL/6 mice to establish a mouse OvCa model. Mice were screened by bioluminescence/ultrasound imaging on day 7 post-injection to confirm adequate tumor uptake (≥ 5 mm mean diameter or $\geq 1 \times 10^6$ photons $\text{s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$) so that each group had equivalent baseline tumor burden (mean $\pm$ SD). The mice were then randomly divided into four groups: control (Ctrl, treated with PBS, $n = 6$), BMS (15 mg/kg, intraperitoneal administration every other day, starting on the 8th day after tumor-bearing, $n = 6$), cis-platin (DDP, 2 mg/kg, intraperitoneal administration once on the 14th day after tumor-bearing, $n = 3$; Qilu Pharmaceutical Company Limited), and combined group (DDP and BMS, $n = 3$). Body weight and abdominal distention were monitored daily. On the 14th day after tumor-bearing, three mice in the Ctrl and BMS groups were randomly selected to sacrifice for the study of the effects of BMS on CD8⁺Tsen cells. The remaining mice in each group were sacrificed and dissected on the 28th day. The spleen, ascites, and inguinal-draining lymph nodes (IDLNs) were collected, corresponding single-cell suspensions were prepared, and CD8⁺Tsen cells were detected by FACS. A parallel survival experiment was performed ($n = 5$ in each group). The administration of BMS continued for 6 w until the abdomen of the mice in the Ctrl group was significantly swollen. The weight and status of the mice were monitored daily. Survival time points were set when the weight reached a cutoff value of 35 g due to ascites accumulation [32]. All mice were anesthetized with 2% isoflurane for 10 min and sacrificed by cervical dislocation. During testing, the investigators responsible for data acquisition were blinded to group allocation.

Bioluminescence imaging (BLI)

ID8-luc-P53^{-/-} cells (3×10^6 cells/200 $\mu\text{l}/\text{mouse}$) were intraperitoneally injected into C57BL/6 mice to establish a metastatic mouse OvCa model. Bioluminescence imaging (BLI) was performed once a week to monitor tumor development. Mice were intraperitoneally injected with D-luciferin (150 mg/kg). 10 min later, they were anesthetized with sevoflurane and placed in an IVIS small animal imaging system to acquire bioluminescence signals [33].

Bioinformatics analysis

For RNA-seq, we incorporated paired RNA-seq data (CD8⁺T + ID8 group, $n = 1$; CD8⁺T + ID8 + MATES group, $n = 1$). After quality control, a total of 28,988 detectable genes were obtained. Subsequently, edgeR v3.40 was used to perform differential analysis on the RNA-seq count matrix. The raw counts were structured into a DGEList object, followed by TMM normalization using calcNormFactors to correct for sequencing depth variations. A fixed dispersion strategy was applied, setting the common dispersion to 0.1. The exactTest (exact negative binomial test in edgeR) was then employed to compare gene expression levels between the two groups, with multiple testing corrected by the Benjamini–Hochberg method. Differentially expressed genes (DEGs) were identified based on the criteria of $|\log_2\text{FC}| > 1$ and $p < 0.05$. Next, the cpm function was used to transform TMM-normalized counts into log₂-CPM values, and genes with no expression variation across all samples were filtered out to reduce noise. PCA analysis (centered and scaled) was performed using prcomp, and sample group information was mapped to the dimensionality reduction results. Finally, GSEA enrichment analysis was conducted using the fgsea package.

Datasets GSE235931, GSE118848 and GSE213243 in the GEO database were analyzed. The GSE235931 dataset comprises tumor samples from 8 ovarian cancer patients (aged 39–77 years) with diverse histological subtypes, including specimens collected from both primary and metastatic sites [34]. The GSE213243 dataset includes samples from 1 high-grade serous ovarian cancer (HGSOC) patient (aged 53 years), derived from ascites and tumor lesions [35]. The GSE118828 dataset consists of primary and metastatic tumor samples from 9 HGSOC patients at varying disease stages [36]. The GSE235931 and GSE118828 datasets were subjected to unified quality control (QC) and batch effect correction, followed by integrative analysis using Seurat v4.3. A total of 1346 CD8⁺T cells were captured (821 cells from primary sites and 525 cells from omental tissues). Differential expression analysis of omental CD8⁺T cells identified 180 DEGs using the FindMarkers function (Wilcoxon rank-sum test, $|\log_2\text{FC}| > 1$, and $p < 0.05$). Gene set enrichment analysis (GSEA) was performed using the fgsea package, FA metabolism-related pathway enrichment was analyzed via the GSVA package, and Gene Ontology (GO) analysis was conducted with the clusterProfiler package. The GSE213243 dataset was similarly processed with unified QC and batch effect correction, followed by integrative analysis using Seurat v4.3. Differential gene expression was assessed using the FindMarkers function.

Statistics analysis

Statistical analysis was performed using GraphPad Prism 9.5 software. One-way ANOVA was used to compare multiple groups. Statistical differences between the two groups were assessed using an unpaired *T*-test. The results of IHC staining and lipofuscin detection were analyzed using two-factor ANOVA. Levene's test revealed no significant heterogeneity of variance across groups. Survival analysis was conducted using the log-rank test (also known as the Mantel–Cox test), and the data were presented as median survival time (MS). Data are presented as mean $\pm$ SD. $p < 0.05$ is considered statistically significant.

RESULTS

The adipocyte-rich tumor microenvironment in OvCa is positively correlated with CD8⁺T cell senescence

To evaluate whether adipocyte-rich TME contributes to the senescence of CD8⁺T cells in OvCa, we first analyzed the single-cell RNA sequencing (scRNA-seq) data of CD8⁺T cells in primary tumors ($n = 6$) and omental metastatic tumors ($n = 7$) in 13 OvCa patients (Fig. S1A–C) in the GEO database. The senescence-related pathways in CD8⁺T cells were up-regulated in omental metastatic tumors compared with primary tumors (Fig. 1A). We also analyzed the scRNA-seq data of CD8⁺T cells in primary tumors and ascites

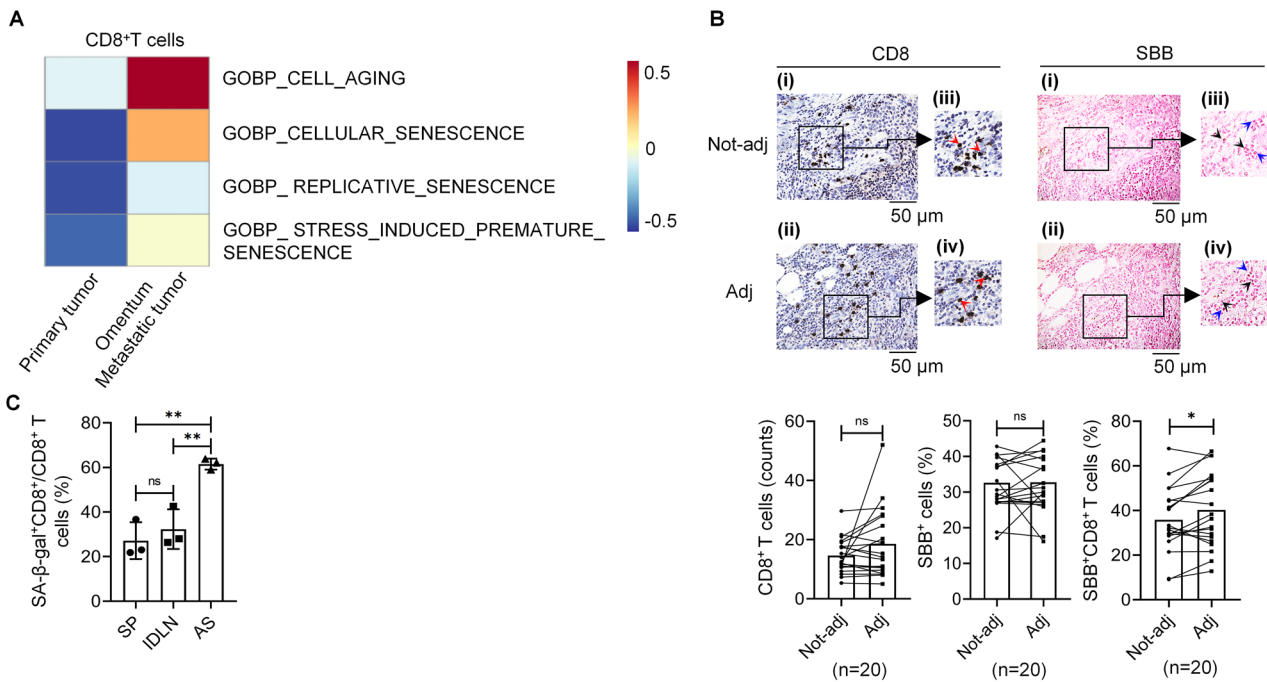


Fig. 1 The adipocyte-rich tumor microenvironment in OvCa is positively correlated with CD8⁺ T cell senescence. **A** GSEA pathway enrichment scores in primary and omental metastatic tumors in OvCa patients (dataset GSE235931 in GEO database, $n = 8$; GSE118828 in GEO database, $n = 5$). **B** Comparison of CD8⁺ Tsen cells in the areas (i) away from (Not-adj) or (ii) adjacent to (Adj) adipose tissue in the specimens of HGSOC patients ($n = 20$). (iii) and (iv) show enlarged images of the indicated areas. Red arrows: representative CD8⁺ T cells. Blue arrows: representative senescent (SBB⁺) cells. Black arrows: representative CD8⁺ Tsen cells. Magnification x200. Each point in the statistical chart represents the mean of the corresponding data in each specimen. **C** The proportion of senescent CD8⁺ T cells in the spleen, inguinal draining lymph nodes, and ascites of tumor-bearing 4w OvCa mice. Data are presented as the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, ns, not significant.

from an HGSOC patient (Fig. S1D–F) in the GEO database. The expression of senescence-related genes, including KLRG1, CDKN1A (P21), TP53 (P53), RB1, and GLB1 [8, 37] of CD8⁺ T cells in ascites was significantly higher than those in primary tumors (Fig. S1G).

Next, we used lipofuscin, a marker of cell senescence [30, 38], to identify CD8⁺ Tsen cells by SBB staining in serial section of HGSOC patients (FIGO Stage III, $n = 20$, age 37–74). To exclude the interference of senescence caused by aging, we compared the distribution of CD8⁺ Tsen cells away from (Not-adj) or adjacent to (Adj) adipose tissue in the same specimen, not in different specimens (Fig. 1B). There was no difference in the number of CD8⁺ T cells (red arrow) and SBB⁺ cells (blue arrow) between the two areas. However, there were more SBB⁺CD8⁺ cells in the Adj area than the Not-adj area (Black arrow, $p < 0.05$), suggesting CD8⁺ T cell senescence is related to adipocyte-rich TME in HGSOC patients. To validate this conclusion, we performed co-staining of SBB and CD8 on the same tissue section, yielding consistent results (Fig. S1H).

Next, we used the EOC mouse model to investigate whether the adipocyte-rich TME exacerbates the formation of CD8⁺ Tsen in ovarian cancer. We employed SA-β-gal staining and flow cytometry to detect the proportion of CD8⁺ Tsen cells in the spleen (SP) and inguinal-draining lymph nodes (IDLN), as well as the ascites (AS) of the same EOC mouse. The results showed that, compared with SP and IDLN, the percentage of CD8⁺ Tsen cells in AS was significantly increased (Fig. 1C and Fig. S1I). These findings suggest that the adipocyte-rich TME in OvCa is positively correlated with the level of CD8⁺ Tsen cells.

MATES aggravates the formation of CD8⁺ Tsen cells induced by OvCa cells

First, we established an in vitro CD8⁺ Tsen-induced cell model [39]. Specifically, CD8⁺ T cells derived from the spleens of tumor-

bearing mice (2 w after the tumor implantation) were co-cultured with mouse ovarian cancer cell line ID8 cells. The proportion of CD8⁺ Tsen cells was detected by SA-β-gal staining using flow cytometry, and the expression levels of the senescence marker genes P21 and P53 were assessed by RT-PCR. The results of SA-β-gal staining showed that the proportion of CD8⁺ Tsen cells was increased after co-culture with ID8 cells for 24 h and 48 h, with the most significant increase observed at 48 h (Fig. 2A). Moreover, the proportion of CD8⁺ Tsen cells did not increase further at 72 h compared with 48 h of co-culture, suggesting that 48 h is the optimal time point for inducing senescence in vitro model (Fig. S2B). Meanwhile, RT-PCR results indicated that after co-culture with ID8 cells for 48 h, the mRNA levels of P21 and P53 in CD8⁺ T cells were significantly upregulated (Fig. 2B), which is consistent with the typical characteristics of senescent cells.

To investigate the impact of an adipocyte-rich TME on tumor-induced CD8⁺ T cell senescence, based on our CD8⁺ Tsen-induced cell model, we used MATES derived from OvCa mice to simulate an adipocyte-rich TME (Fig. 2C). We then detected the proportion of CD8⁺ Tsen cells by SA-β-gal staining with flow cytometer and assessed the expression levels of P21 and P53 using RT-PCR. The SA-β-gal staining results showed that, compared with the CD8+ID8 group, MATES could exacerbate the formation of CD8⁺ Tsen cells induced by ID8 cells (Fig. 2D). RT-PCR results showed that after treatment with MATES, the mRNA levels of P21 and P53 in CD8⁺ T cells were significantly upregulated (Fig. 2E).

To explore the reasons why an adipocyte-rich TME exacerbates tumor-induced CD8⁺ T cell senescence, we first treated the co-culture system with MATES and performed RNA sequencing (RNA-seq). The sequencing data were analyzed by principal component analysis (PCA) for cluster visualization (Fig. S2C) to exclude outliers, followed by differential gene expression analysis (Fig. S2D), and subsequently subjected to Gene Set Variation Analysis (GSVA) for

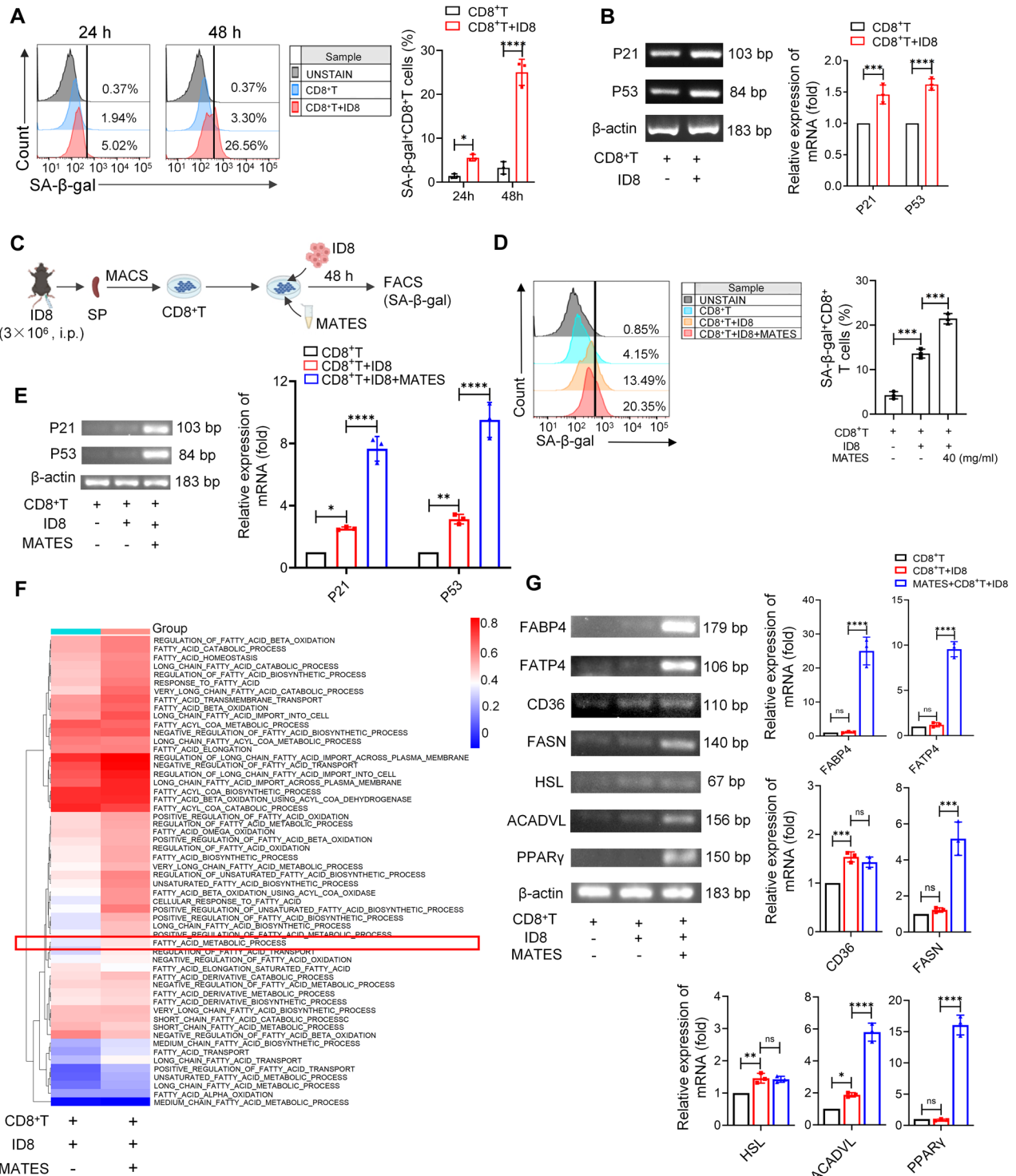
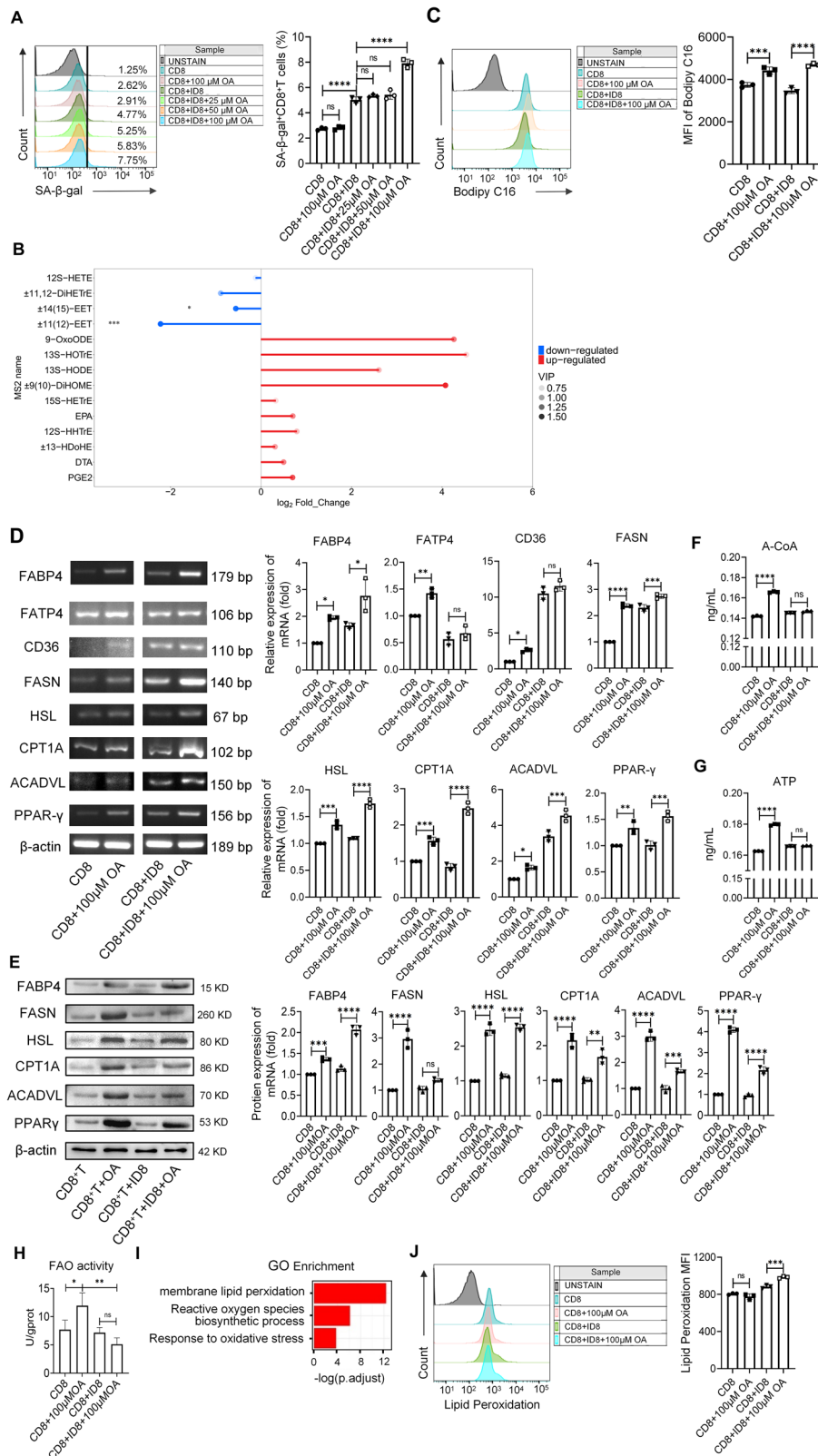


Fig. 2 **MATES aggravates the formation of CD8⁺Tsen cells induced by OvCa cells.** **A** The proportion of CD8⁺Tsen cells after co-culture with ID8 cells was assessed by SA-β-gal staining. **B** The mRNA levels of p21 and p53 in CD8⁺T cells treated with ID8. **C** Experimental scheme for the induction and detection of Tsen cells. **D** The proportion of CD8⁺Tsen cells after MATES treatment was assessed by SA-β-gal staining. **E** The mRNA levels of p21 and p53 in CD8⁺T cells treated with MATES. **F** Analysis of FA metabolism-related signaling pathways based on RNA-seq results. ssGSEA was used to score the FA metabolic pathways. **G** The mRNA levels of FA metabolism-related genes in CD8⁺T cells. MATES, mouse adipocyte tissue extracts; MACS, magnetic cell sorting. For mRNA detection, the relative expression of each gene was calculated using β-actin as the internal reference. Data are presented as the mean ± SD of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns, not significant.



pathway enrichment. The results showed that, compared with co-cultured CD8⁺T cells and ID8 cells, the FA metabolic pathway (red arrow) in CD8⁺T cells was significantly upregulated after treatment with MATES (Fig. 2F). We then performed RT-PCR assay. The results showed that FA binding protein 4 (FABP4), FA transport protein 4

(FATP4), FA synthase (FASN), acyl coenzyme-A dehydrogenase very long chain (ACADVL), and the FA nuclear transcription receptor, peroxisome proliferator-activated receptor gamma (PPAR γ), were significantly upregulated, with the most significant increase observed in FABP4 (Fig. 2G). These data suggest that

Fig. 3 OA exacerbates the formation of CD8⁺Tsen cells induced by OvCa cells through promoting lipid peroxidation. **A** Detection the proportion of CD8⁺Tsen cells when CD8⁺T cells and ID8 cells were cocultured and treated with OA. **B** Matchstick analysis of lipid metabolic products ($n = 8$). **C** Quantification of the FA content by a Bodipy-FL-C16 capture assay in CD8⁺T cells after treatment with ID8 and OA. **D** The mRNA levels of FA metabolism-related genes in CD8⁺T cells. Intracellular levels of A-CoA (**E**) and ATP (**F**) in CD8⁺T cells after treatment with ID8 and OA were measured by ELISA. **G** Intracellular levels of FAO activity in CD8⁺T cells after treatment with ID8 and OA was evaluated with a colorimetric assay kit. **H** GO pathway enrichment scores in primary tumors and omental metastases from OvCa patients (dataset GSE235931 from the GEO database, $n = 8$; GSE118828 from the GEO database, $n = 5$). **I** Intracellular oxidized lipid content in CD8⁺T cells after treatment with ID8 and OA was measured using Bodipy C11 staining. For mRNA detection, the relative expression of each gene was calculated using β -actin as the internal reference. **J** Intracellular oxidized lipid content in CD8⁺T cells after treatment with ID8 and OA was measured using Bodipy C11 staining. Data are presented as the mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant.

MATES significantly upregulate FA metabolism in CD8⁺T cells, thereby promoting the formation of CD8⁺Tsen cells.

OA exacerbates the formation of CD8⁺Tsen cells induced by OvCa cells through promoting lipid peroxidation

To further investigate the mechanisms by which the adipocyte-rich TME exacerbates tumor-induced CD8⁺Tsen in OvCa, we first used CD8⁺Tsen-induced cell model to conduct a dose-effect experiment. OA (an 18 carbon FA), a representative unsaturated FA, is the most abundant FA in the malignant ascites of OvCa patients [40]. Different concentrations of OA were added into above CD8⁺Tsen-induced cell model and then the proportion of CD8⁺Tsen cells was detected by SA- β -gal staining with flow cytometer. The results showed that compared with the CD8 + ID8 group, there was no significant change in the proportion of CD8⁺Tsen cells when the concentration of OA was 25 μ M or 50 μ M. However, when the concentration of OA reached 100 μ M, the proportion of CD8⁺Tsen cells was significantly increased (Fig. 3A). The results indicate that OA can exacerbate the formation of CD8⁺Tsen cells induced by tumor cells in the adipocyte-rich TME of ovarian cancer.

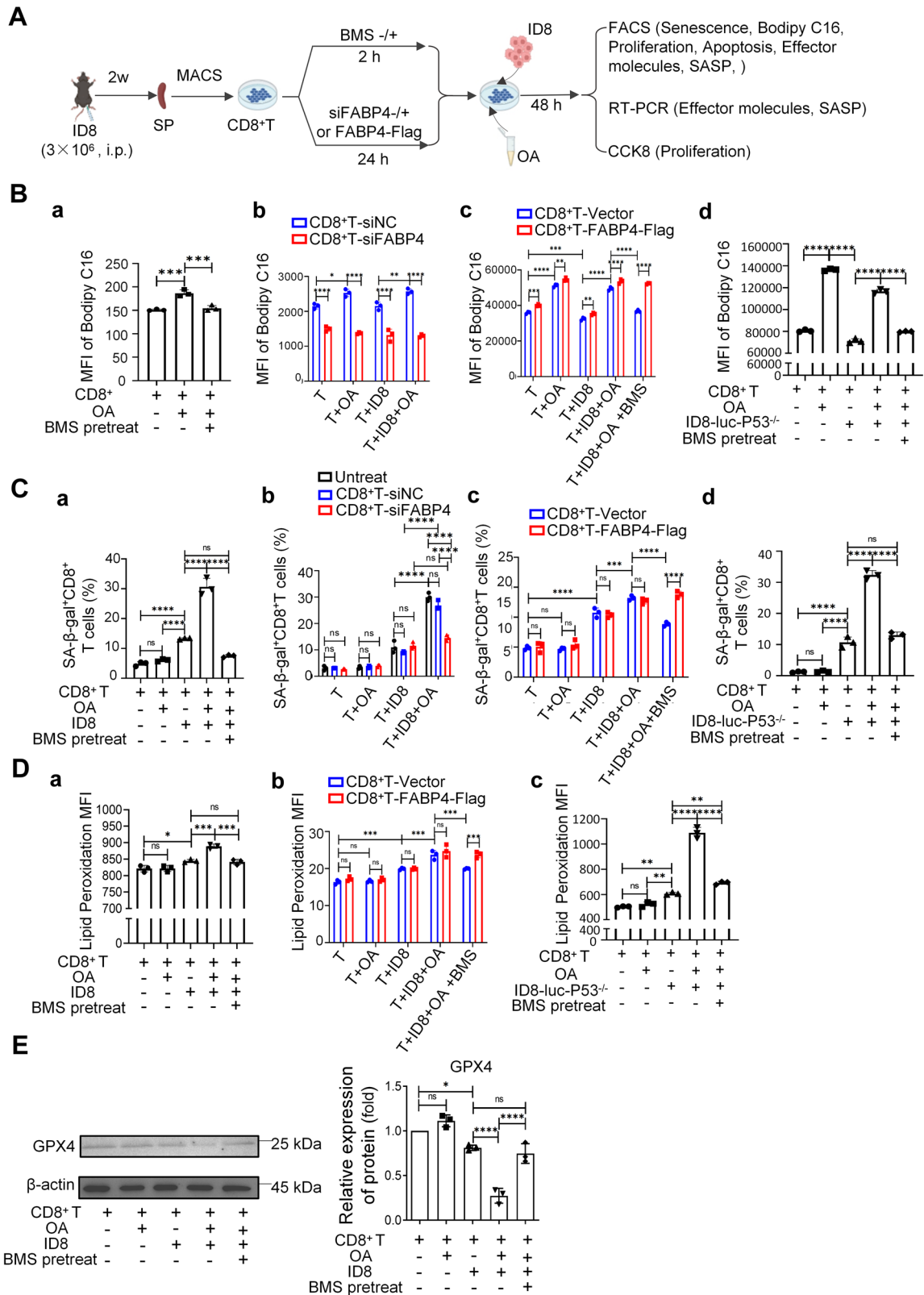
To further explore the mechanisms by which OA exacerbates the formation of CD8⁺Tsen induced by ID8 cells, we used CD8⁺Tsen-induced cell model and treated it with 100 μ M OA. We first performed lipidomics sequencing, followed by principal component analysis (PCA) for cluster visualization (Fig. S3A). Potential outliers in the dataset were identified using the 95% confidence interval of principal component scores as the threshold. Subsequently, differential metabolite analysis was conducted. The results revealed that compared with the CD8 + ID8 group, OA treatment significantly downregulated oxidized lipids $\pm 11(12)$ -EET and $\pm 14(15)$ -EET in CD8⁺T cells, while lipid peroxidation markers 9-OxoODE, 13S-HODE, 13S-HOTRe, and $\pm 9(10)$ -DiHOME showed an upward trend (Fig. 3B). $\pm 11(12)$ -EET and $\pm 14(15)$ -EET are enzymatically generated oxidized lipids, whose elevation is often associated with enhanced fatty acid oxidation (FAO) capacity [41]. These results suggest that in the presence of ID8, OA fails to enhance FAO capacity in CD8⁺T cells but may instead promote their lipid peroxidation. To validate the lipidomics findings, we then assessed intracellular FA content in CD8⁺T cells using the Bodipy C16 uptake assay. Expression of FA metabolism-related genes was measured by RT-PCR, while levels of acetyl-CoA (A-CoA) and ATP were quantified by WB and ELISA, respectively. The fatty acid oxidation (FAO) activity in CD8⁺T cells was measured using a fatty acid oxidation assay kit. The results showed that, compared with the CD8 + ID8 group, the intracellular FA content of CD8⁺T cells in the CD8 + ID8 + OA group was significantly increased (Fig. 3C). RT-PCR and WB analyses showed that the expression levels of FA uptake (FABP4), synthesis (FASN), lipolysis (HSL), and oxidation (CPT1A, ACADVL) genes in CD8⁺T cells were all elevated (Fig. 3D and Fig. 3E). The ELISA results showed that, compared with the CD8 group, the levels of A-CoA, ATP and FAO activity in CD8⁺T cells were significantly elevated in the CD8 + OA group. However, no significant changes were observed in the levels of A-CoA (Fig. 3F), ATP (Fig. 3G), or FAO activity (Fig. 3H) in

CD8⁺T cells of the CD8 + ID8 + OA group compared with the CD8 + ID8 group. These results suggest that, in the presence of ID8 cells, the uptake of OA by CD8⁺T cells is not used for energy production.

To investigate the fate of OA after its uptake by CD8⁺T cells, we performed GO pathway enrichment analysis using the scRNA-seq data of CD8⁺T cells from primary tumors ($n = 6$) and omental metastases ($n = 7$) of 13 ovarian cancer patients in the GEO database. Compared with primary tumors, we found that in omental metastases, CD8⁺T cells exhibited upregulation of membrane lipid peroxidation, reactive oxygen species biosynthetic process and response to oxidative stress signaling pathways (Fig. 3I and Fig. S3B-C). Additionally, we detected the levels of lipid peroxidation using the Bodipy C11 uptake assay with flow cytometer. The results showed that compared with the CD8 group, the CD8⁺T cells in the CD8 + OA group did not exhibit increased oxidized lipids. However, compared with the CD8 + ID8 group, the CD8⁺T cells in the CD8 + ID8 + OA group displayed a significant increase in oxidized lipids (Fig. 3J). These results suggest that in the adipocyte-rich TME of ovarian cancer, OA exacerbates ID8-induced CD8⁺Tsen cells by promoting lipid peroxidation.

In vitro inhibition of OA uptake mediated by FABP4 reduces the formation of OvCa-induced CD8⁺Tsen cells

As shown in Figs. 2G and 3D, the most significantly upregulated gene in CD8⁺T cells after treatment with MATES and OA is FABP4. FABP4 is one of the major FA transport molecules, responsible for transporting long-chain FAs (containing 13–21 carbon atoms, including OA [42]). To investigate the effects of inhibiting OA uptake on tumor cell-induced CD8⁺T cells senescence in vitro, we used the model and pre-treated CD8⁺T cells with the FABP4 inhibitor BMS309403 (BMS) or FABP4-targeting small interfering RNA (siFABP4) to specifically suppress OA uptake (Fig. 4A and Fig. S4A). We detected the intracellular FA level of CD8⁺T cells using the Bodipy C16 uptake assay with flow cytometer, determined the proportion of CD8⁺Tsen cells using SA- β -gal staining with flow cytometer, assessed the level of lipid peroxidation using the Bodipy C11 uptake assay with flow cytometer, and evaluated Glutathione peroxidase 4 (GPX4) expression levels using western blotting. GPX4 is a key lipid peroxidase that functions as a central regulator of cellular redox homeostasis, with its expression levels exhibiting a robust negative correlation with intracellular lipid peroxidation [43]. The result showed that compared with OA treatment alone, in BMS or siFABP4 treatment group, in CD8⁺T cells, intracellular FA levels were significantly reduced (Fig. 4Ba-b and Fig. S4Ba-b). Meanwhile, the proportions of CD8⁺Tsen were reduced (Fig. 4Ca-b and S4Ca-b). In terms of mechanism, compared with OA treatment alone, in BMS or siFABP4 treatment group, in CD8⁺T cells, lipid peroxidation levels were significantly reduced (Fig. 4Da and Fig. S4Da), and GPX4 protein expression was upregulated (Fig. 4E). These results suggest that inhibiting OA uptake in vitro can reduce ID8-induced CD8⁺Tsen cells by attenuating lipid peroxidation.



BMS, as reported by Masato Furuhashi et al., shows preferential binding to FABP4 over FABP5, with higher affinity and selectivity for FABP4 [42]. To validate that BMS inhibits CD8⁺T cell fatty acid (FA) uptake by blocking FABP4, we overexpressed FABP4 in CD8⁺T cells and subsequently measured intracellular FA content,

lipid peroxidation levels, and the proportion of senescent CD8⁺T cells in the co-culture system via flow cytometry (Fig. S4E). The results showed that, compared with BMS treatment, overexpression of FABP4 abolished the effects of BMS, including preventing CD8⁺T cell uptake of OA (Fig. 4Bc and Fig. S4Bc),

Fig. 4 **In vitro inhibition of OA uptake mediated by FABP4 reduces the formation of OvCa-induced CD8⁺Tsen cells.** **A** Experimental scheme. **B** CD8⁺T cells were pretreated with BMS for 2 h (**a**), transfected with siFABP4 for 24 h (**b**), transfected with FABP4 over-expression plasmid for 24 h (**c**) or co-cultured with ID8-luc-p53^{-/-} cells for 24 h (**d**), and intracellular FA content was quantified using the Bodipy C16 uptake assay. **C** The proportion of CD8⁺Tsen cells was measured after pretreatment of CD8⁺T cells with BMS for 2 h (**a**), siFABP4 transfection for 24 h (**b**), FABP4 over-expression plasmid transfection for 24 h (**c**) or co-cultured with ID8-luc-p53^{-/-} cells for 24 h (**d**). **D** Intracellular oxidized lipid content in CD8⁺T cells after BMS treatment (**a**), FABP4 over-expression plasmid transfection for 24 h (**b**) or co-cultured with ID8-luc-p53^{-/-} cells for 24 h (**c**) was measured using Bodipy C11 staining. **E** Protein expression of β -actin and GPX4 in CD8⁺T cells after BMS treatment was assessed by WB. β -actin was used as the internal control to calculate the relative expression level of GPX4. For proteins detection, the relative expression of each protein was calculated using β -actin as the internal reference. MFI mean fluorescence intensity, FACS fluorescence-activated cell sorting, MACS magnetic-activated cell sorting, BMS BMS309403. Data are presented as the mean $\pm$ SD of three independent experiments. ** p < 0.01, *** p < 0.001, **** p < 0.0001, ns not significant.

attenuating OA-aggravated the formation of senescent CD8⁺T cell by induced ID8 cells (Fig. 4Cc and Fig. S4Cc), and reducing lipid peroxidation (Fig. 4Db and Fig. S4Db). These results indicate that, in the presence of tumor cells, BMS specifically inhibits FABP4 to block CD8⁺T cell uptake of OA, reduce lipid peroxidation, and thereby decrease senescent CD8⁺T cell formation.

In vitro inhibition of OA uptake mediated by FABP4 enhances anti-tumor immunity

CD8⁺T cells initiate their activation process in peripheral immune organs, and only after antigen stimulation can they exert the effector function [44]. Tsen cells directly inhibit the function of effector T cells [10]. To further investigate the impact of inhibiting OA uptake on reducing the formation of CD8⁺Tsen and its resulted effect on anti-tumor immunity, we assessed the proliferative capacity of CD8⁺T cells using CCK8 and Ki67 staining, measured the expression levels of effector molecules (GZMB, FasL, and IFN- γ) using RT-PCR and flow cytometry, as well as the expression levels of key SASP (senescence-associated secretory phenotype) factors such as IL-6 and inhibitory cytokines like IL-10 in CD8⁺T cells using flow cytometry. The results from CCK8 and Ki67 assays showed that when co-cultured with ID8 cells, the proliferation of CD8⁺T cells was enhanced under antigen stimulation but was subsequently abrogated by OA treatment. This OA-induced abrogation was effectively reversed by BMS or siFABP4 (Fig. 5Aa-b and Fig. S5Aa-b). RT-PCR and flow cytometry analysis indicated that when co-cultured with ID8 cells, the mRNA levels (Fig. S5Ba-b) and the mean fluorescence intensity (MFI) of effector molecules such as GZMB, FasL, and IFN- γ were significantly increased. These increases were inhibited by OA treatment and were ultimately reversed by pre-treatment with BMS or siFABP4 (Fig. 5Ba-b and Fig. S5Ca-b). The MFI levels of IL-6 and IL-10 (Fig. 5Ba-b and Fig. S5Ca-b) showed similar changes to those observed in CD8⁺Tsen cells in the Fig. 4C. Additionally, we assessed the viability of ID8 cells using CCK8 and Ki67 staining. The results showed that compared with the co-culture group, OA significantly increased the survival of ID8 cells and reduced apoptosis in ID8 cells (Fig. 5Ca-c, Fig. S5Da-b and S5E). These changes were reversed by pre-treatment of CD8⁺T cells with BMS or siFABP4. The above data suggest that inhibiting OA uptake partially improves the anti-tumor immune response, likely due to the reduced formation of CD8⁺Tsen cells.

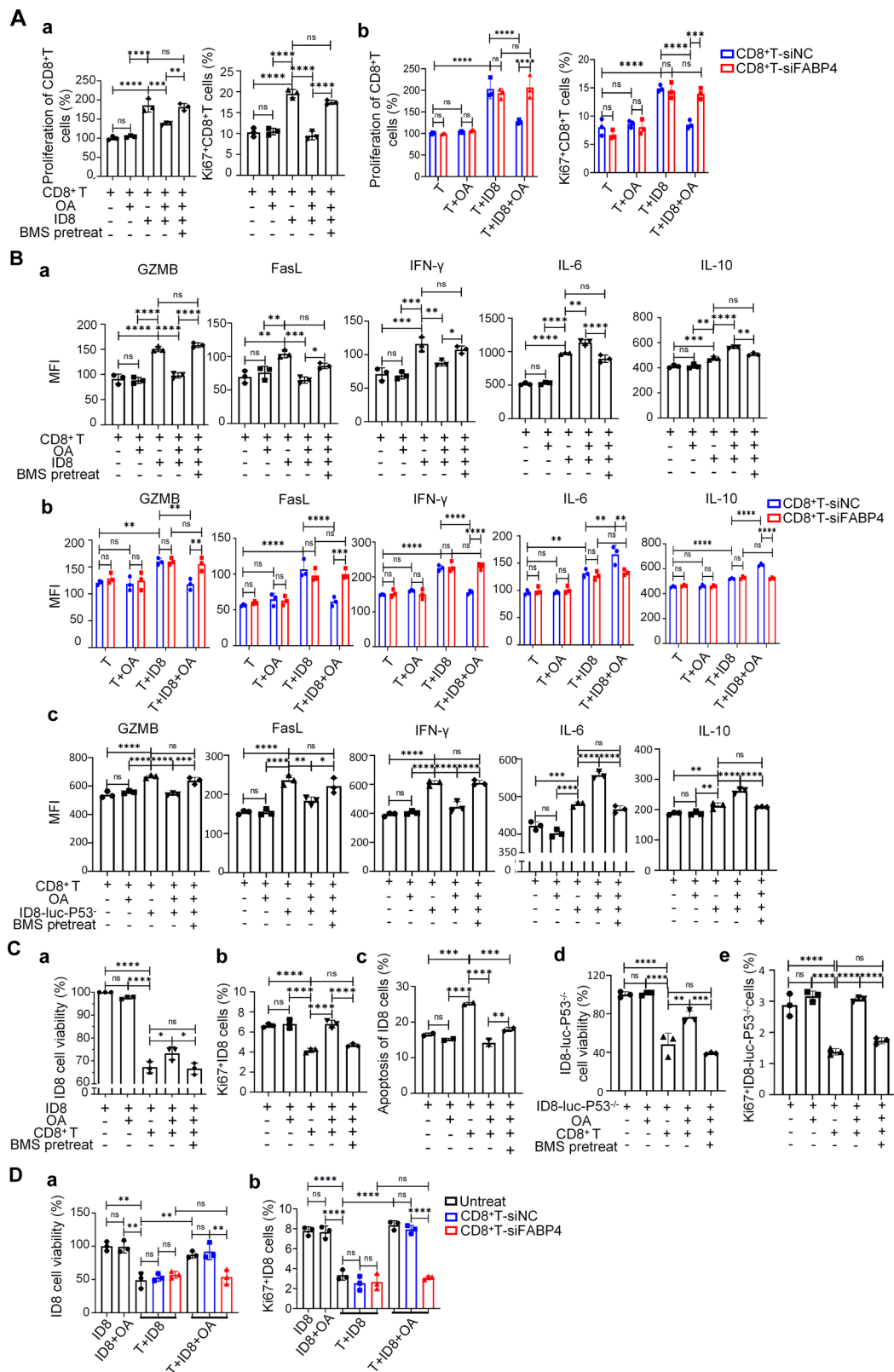
To validate these findings, we generated an ID8-luc-p53^{-/-} cell line using CRISPR/Cas9 technology, and establish an ID8-luc-p53^{-/-} epithelial ovarian cancer (EOC) mouse model (Fig. S5F). P53 is a commonly deleted gene in ovarian cancer, and 96% of HGSOE cases harbor Trp53 mutations [45]. We used flow cytometry and CCK-8 assays to detect the relevant indicators in Figs. 4, 5. Flow cytometry results showed that, compared with control CD8⁺T cells, ID8-luc-p53^{-/-} significantly induced CD8⁺T cell senescence, OA further exacerbated the CD8⁺T cell senescence induced by ID8-luc-p53^{-/-} cells (Fig. 4Cd and S4Cd), promoted the expression of IL-6 and IL-10 and reduced the expression levels of effector molecules, including GZMB, FasL, and IFN- γ (Fig. 5Bc and Fig. S5Cc). Concurrently, OA elevated lipid peroxidation levels in CD8⁺T cells

when ID8-luc-p53^{-/-} cells were present (Fig. 4Dc and S4Dc). CTL killing assay results showed that OA treatment significantly inhibited the ability of CD8⁺T cells to kill ID8-luc-p53^{-/-} cells, while BMS treatment reversed this effect (Fig. 5Cd-e and S5Dc). These results further indicate that inhibition of OA uptake reduces the formation of ovarian cancer-induced CD8⁺Tsen cells and enhances anti-tumor immunity.

In vivo intervention of FA uptake partially alleviates CD8⁺T cell senescence and improves anticancer immunity of OvCa mice combined with chemotherapy

To verify the effects of in vivo intervention of FA uptake on reducing CD8⁺Tsen cells, we established an OvCa metastasis mouse model. According to the protocol illustrated in Fig. 6A, we treated the mice with BMS, a FABP4 inhibitor. After 1 w of treatment, the mice were sacrificed to collect ascitic CD8⁺T cells. We then used the Bodipy C16 uptake assay, SA- β -gal staining, RT-PCR, and western blotting to assess BMS effects on CD8⁺Tsen cells (Fig. 6A). The gating strategy for ascitic CD8⁺T cells is shown in Fig. S6B. Compared with the Ctrl group, mice in the BMS group showed significant decreases in the same indexes as the in vitro studies, including the MFI level of Bodipy in CD8⁺T cells (Fig. 6B); the proportion of CD8⁺Tsen cells (Fig. 6C); the mRNA levels of P21 and P53 (Fig. 6D) and the protein expression of GPX4 in CD8⁺T cells (Fig. 6E). Meanwhile, the mRNA level of IFN- γ , FasL, and GZMB of CD8⁺T cells was significantly increased in the BMS group than in the Ctrl group (Fig. 6F). These results indicate that BMS treatment reduces the formation of CD8⁺Tsen in vivo by inhibiting FA uptake and thereby decreasing lipid peroxidation.

To further explore the feasibility of decreasing CD8⁺Tsen formation by inhibiting FA uptake, and enhancing anti-cancer immunity in combination with chemotherapy, we used above OvCa metastasis mouse model, and administered BMS and DDP treatment according to the protocol shown in Fig. 6G. Subsequently, we employed flow cytometry to assess the senescent proportion and effector functional molecules of CD8⁺T cells, as well as the proliferation status and functional characteristics of CD4⁺T cells. Concurrently, we utilized small-animal imaging to monitor tumor progression and conducted survival assays. BMS could alleviate chemoresistance in OvCa [26]. Hence, DDP was administered only once to exclude the interference of chemoresistance. The mice were sacrificed 4 w after tumor cell implantation to observe the efficacy of the treatments (Fig. 6G). There were much more and bigger tumor nodules on the abdominal wall (Fig. 6H, red box a, yellow arrow) of mice in the Ctrl group than those in the other groups. In the mice of the combined group, the abdominal wall was smooth and no tumor nodules were found. Compared with the mice in the Ctrl group, the number of tumor nodules (red box b, orange arrow) on the outer wall of the intestine of mice in the BMS group and DDP group was reduced (Fig. 6H), and the mice in the combined group had the fewest nodules. Except for the CTRL group, no overt hemorrhagic ascites was observed in mice from other treatment groups (Fig. S6A). Small-animal imaging analysis revealed that at 4 w post tumor inoculation, the tumor cell burden was significantly lower in the



combination therapy group compared with other groups (Fig. 6I). Meanwhile, flow cytometry results showed that the proportion of CD8⁺Tsen cells in the spleen, ascites, and IDLNs in the mice of the combined group was significantly lower than in the other groups (Fig. 6J and Fig. S6C). This finding was further validated in the ID8-

luc-P53^{-/-} mouse model, where the combination therapy group exhibited the lowest proportion of senescent CD8⁺T cells in the spleen, ascites, and IDLNs (inguinal draining lymph nodes) compared with other groups, along with the highest levels of CD8⁺T cell effector functional molecules GZMB, FASL, and IFN-γ in

Fig. 5 In vitro inhibition of OA uptake mediated by FABP4 enhances anti-tumor immunity. **A** CD8⁺ T-cell proliferation and Ki67 expression were assessed by CCK-8 assay and flow cytometry after treatment with BMS (**a**), siFABP4 transfection, or FABP4-over-expression-plasmid transfection (**b**). **B** Mean fluorescence intensity (MFI) of GZMB, FasL, IFN- γ , IL-6, and IL-10 in CD8⁺T cells after treatment with BMS (**a**), siFABP4 transfection (**b**), or FABP4-over-expression-plasmid transfection (**c**). **C** ID8 cell proliferation (**a**), Ki67 expression levels (**b**), apoptosis (**c**), ID8-luc-P53^{-/-} cell proliferation (**d**) and proportion of Ki67⁺ID8-luc-P53^{-/-} cells (**e**) after treatment with BMS were assessed using CCK8 assay and flow cytometry. **D** ID8 cell proliferation (**a**) and Ki67 expression levels (**b**) after treatment with siFABP4 were assessed using CCK8 assay and flow cytometry. For mRNA detection, the relative expression of each gene was calculated using β -actin as the internal reference. MFI mean fluorescence intensity, FACS fluorescence-activated cell sorting, MACS magnetic-activated cell sorting, BMS BMS309403. Data are presented as the mean $\pm$ SD of three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant.

ascites, as well as IL-2 levels in CD4⁺ T cells (Fig. 6K-M and Fig. S6D-F). In the parallel survival experiment, the administration of BMS continued for 6 w until the abdomen of the mice in the Ctrl group was significantly swollen. The combination of BMS and DDP significantly extended the survival time of mice (MS: 100 d) compared with the DDP group (MS: 89 d) (Fig. 6N). These results suggested inhibition of FA uptake by BMS partially alleviated CD8⁺T cell senescence, improved anticancer immunity, and prolonged the survival time of OvCa mice combined with chemotherapy.

DISCUSSION

Adipocyte-rich TME is a risk factor for T-cell dysfunction [46, 47]. In the present study, we found that, in vitro, in the absence of tumor cells, OA (the most abundant FA in ascites) can be taken up by CD8⁺T cells to generate ATP through fatty acid oxidation (FAO) without affecting the formation of CD8⁺Tsen. However, in the presence of tumor cells, OA can still be taken up by CD8⁺T cells, but FAO does not occur, and no ATP is produced. Instead, OA increase the formation of CD8⁺Tsen induced by tumor cells through lipid peroxidation (Fig. 7). In tumor-bearing mice, inhibiting FA uptake can decrease the formation of CD8⁺Tsen, improve anti-tumor immunity, and prolong the survival time of ovarian cancer mice treated with BMS plus chemotherapy. These results indicate that FA metabolism, especially uptake of FA, in the adipocyte-rich TME plays a crucial role in the formation of CD8⁺Tsen, and its formation can be decreased and resulted anti-tumor immunity can be enhanced by intervening in FA uptake.

The extracellular long-chain FAs are mainly transported by FA transport molecules including CD36, FABPs and FATPs, which play various roles in T cell functions [48–50]. In the present study, the mRNA level of CD36 in either MATES-treated ID8 cell-induced CD8⁺Tsen cells or ascitic CD8⁺Tsen cells did not show significant differences when compared with their controls, respectively. However, other researchers emphasized the role of CD36 in CD8⁺T cells in cancers. CD36 was enriched in dysfunctional TILs, and FA uptake by CD36 results in reduced secretion of effector molecules such as IFN- γ , GZMB, and TNF in mice with melanoma colon adenocarcinoma [51] and patients with non-small cell lung cancer [48]. This discrepancy may be due to different cancer types.

It is worth mentioning that, in this study, we also used the in vitro CD8⁺Tsen-induced cell model mentioned above to examine the impact of palmitic acid (PA, the second most abundant FA in malignant ascites) on CD8⁺Tsen formation. The results showed that, unlike OA, PA had no significant effects on the induction of CD8⁺Tsen, neither the production of acetyl-CoA (A-CoA), ATP production, nor the occurrence of lipid peroxidation (Fig. S7A–E). The reason for that might be that PA, as a saturated fatty acid, has lower propensity for lipid peroxidation [17]. These suggest that when CD8⁺T cells uptake PA, it is not used for fatty acid oxidation (FAO) to generate energy nor for lipid peroxidation. Instead, it is likely to be stored within the cells in the form of lipid droplets. The reason for such speculation is that the results of the

Bodipy C16 assay showed an increase in intracellular lipid content (Fig. S7B).

Recently, inhibitors targeting FA metabolism have been applied in preclinical studies [52], e.g., CD36 blockers are optionally combined with ICB therapy for melanoma and pancreatic cancer. For HGSOc, chemotherapy is one of the first-line treatments and the efficacy of ICB therapy is poor [22]. Hence, in the animal experiments, we combined chemotherapy with BMS (a FABP4 inhibitor) to reduce CD8⁺T cell senescence by targeting two aspects: cancer cells, the main source that induces CD8⁺T cell senescence, and FA uptake, which aggravated the formation of CD8⁺Tsen cells. In TME, FA uptake facilitates rapid growth and proliferation of cancer cells [53], which may indirectly exacerbate CD8⁺T cell senescence. Therefore, BMS treatment probably decreased the generation of CD8⁺Tsen cells indirectly by affecting cancer cells. BMS can also alleviate carboplatin resistance [26]. The present study proved that BMS enhances anticancer immunity by reducing the generation of CD8⁺Tsen cells and is a good drug for combination with chemotherapy by decreasing the administration frequency of DDP, finally alleviating the side effects. However, the timing, dosage, and frequency of BMS treatment require further exploration. Han et al. have demonstrated that cystine deprivation induces CD36-mediated lipid accumulation and CD8⁺T cell ferroptosis, leading to CD8⁺T cell exhaustion [54]. Nevertheless, the impact of adipocyte rich microenvironment with lipid peroxidation on CD8⁺T cell exhaustion remains unexplored, and the therapeutic efficacy of disrupting this microenvironment in clinical applications warrants further investigation.

Within the tumor microenvironment (TME), lipid peroxidation is regulated by various factors in addition to fatty acids, such as reactive oxygen species (ROS), iron, and glutathione (GSH) [55]. E.g., tumor growth requires substantial energy consumption and simultaneously generates elevated levels of ROS [56]. The elevated ROS can modulate immune cells by interacting with membrane phospholipids, thereby initiating lipid peroxidation reactions that drive cellular senescence [31]. In the TME, tumor cells can also undergo lipid peroxidation, which has complex effects on various immune cells. For example, prostaglandin E2 (PGE2) is a lipid peroxidation product of tumor cells, and PGE2-EP4/EP2 signaling impairs both adaptive and innate immunity in TME by hampering bioenergetics and ribosome biogenesis of tumor-infiltrating immune cells including CD8⁺T cells and macrophages [57]. Tumor cells with lipid peroxidation lack immunogenicity and impair the cross-presentation and anti-tumor ability of DCs [58].

In summary, our study reveals that the adipocyte-rich TME exacerbates the formation of CD8⁺Tsen induced by ovarian cancer cells through promoting lipid peroxidation. We focus on the lipid metabolism reprogramming of CD8⁺T cells and propose therapeutic strategies to mitigate CD8⁺Tsen formation by targeting FA metabolism, thereby enhancing anti-tumor immunity. This study provides insights into targeting dysfunctional T cells for anti-tumor immunotherapy from the perspective of FA metabolism.

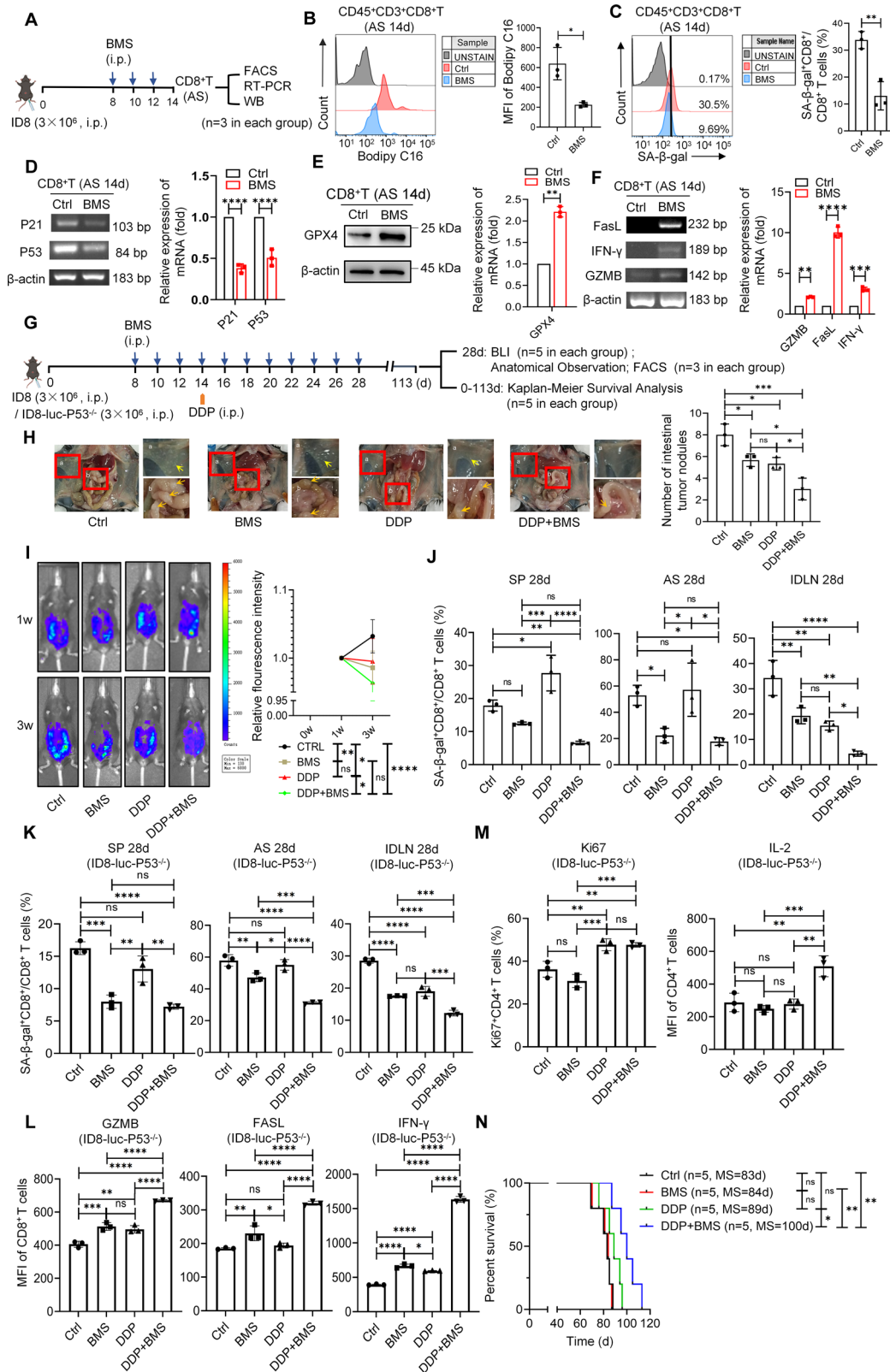


Fig. 6 **In vivo intervention of FA uptake partially alleviates CD8⁺T cell senescence and improves anticancer immunity of OvCa mice combined with chemotherapy.** **A** Experimental scheme to detect the effect of BMS on Tsen cells. **B** Quantifying the FA content of the ascitic CD8⁺T cells in mice of the Ctrl and BMS groups ($n = 3$ each). **C** Proportion of CD8⁺Tsen cells in the ascitic CD45⁺CD3⁺CD8⁺T cells from mice in the Ctrl and BMS group. **D** mRNA levels of P21, and P53 in ascitic CD8⁺T cells. **E** Protein expressions of β -actin, GPX4 in ascitic CD8⁺T cells. β -actin was used as the internal control to calculate the relative expression level of GPX4. **F** The mRNA levels of GZMB, FasL, and IFN- γ of the ascitic CD8⁺T cells. **G** Experimental scheme to detect changes of Tsen cells and a parallel survival experiment after treatments. **H** Representative images and numbers of the abdominal tumor nodules of mice in each group ($n = 3$). Red box a and yellow arrow: representative tumor nodules on the abdominal wall. Red box b and orange arrow: representative tumor nodules on the outer wall of the intestine. **I** Representative BLI images and comparison of OvCa progression in each group ($n = 3$). C57BL/6 mice model established by ID8-luc-P53^{-/-} cells. **J** C57BL/6 mice model established by ID8 cells. **K** C57BL/6 mice model established by ID8-luc-P53^{-/-} cells. **L** Mean fluorescence intensity (MFI) of GZMB, FasL and IFN- γ in CD8⁺T cells from C57BL/6 mice model established by ID8-luc-P53^{-/-} cells. **M** Proliferation (Ki67) and effector function (IL-2) of splenic CD4⁺T cells. **N** Kaplan–Meier analysis of the survival time of mice in each group ($n = 5$) presented by MS (median survival). BMS, BMS309403; DDP, cisplatin; SP, spleen; AS, ascites; IDLN, inguinal-draining lymph nodes. For mRNA detection, the relative expression of each gene was calculated using β -actin as the internal reference. For proteins detection, the relative expression of each protein was calculated using β -actin as the internal reference. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

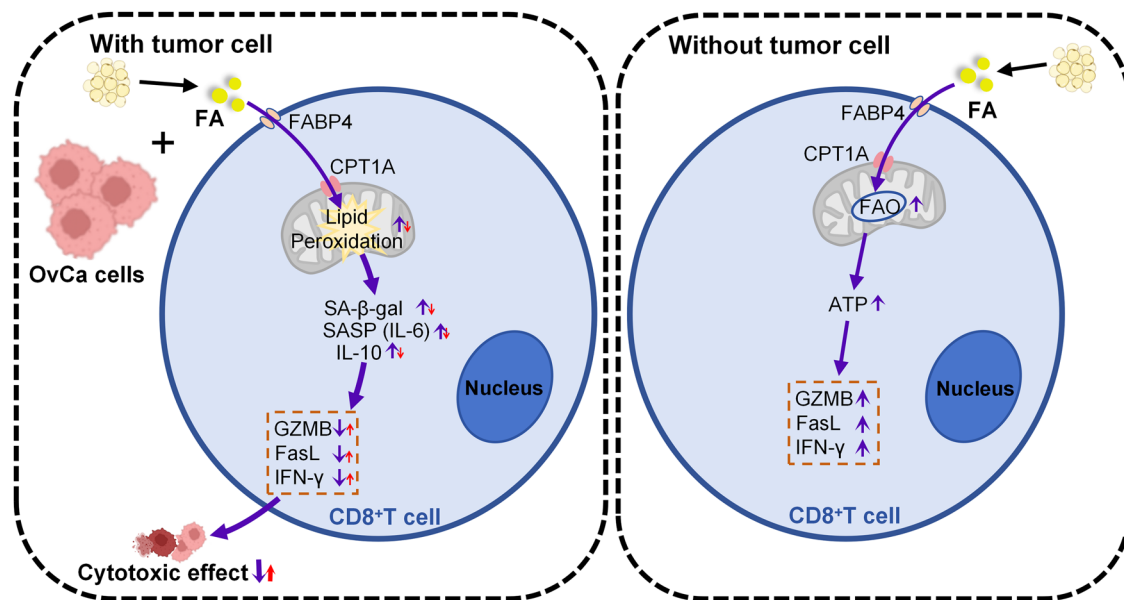


Fig. 7 **The mechanism diagram of OA exacerbating CD8⁺T cell senescence induced by OvCa cells.** In the absence of tumor cells, OA is taken up by CD8⁺T cells and metabolized through FAO to generate ATP, without promoting CD8⁺Tsen. However, in the tumor microenvironment, while CD8⁺T cells still uptake OA, FAO is impaired and ATP production ceases. Instead, OA exacerbates tumor-induced CD8⁺Tsen formation through lipid peroxidation-mediated mechanisms.

DATA AVAILABILITY

All the data are available within the article. The RNA-seq data can be found in Sequence Read Archive (SRA accession PRJNA1181575). Supplementary Information and Source Data file are available from the corresponding author upon reasonable request. The code used in this study has been uploaded as Supplementary Material.

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AUTHOR CONTRIBUTIONS

YCY: conceptualization, data curation, formal analysis, validation, investigation, visualization, methodology, writing—original draft, writing—review and editing. LX: conceptualization, data curation, formal analysis, writing—review and editing. QXL: formal analysis, validation, investigation and visualization. ZHK: validation, investigation, visualization, methodology and writing—review. LXY, WB, LMT, LZ, DW, CSQ, OYYQ, FFF: investigation. HTH, LZ, WHX, ZXY, LJR, ZH, SYM, LCY, LJZ, GHY: methodology. XSW: supervision, funding acquisition. GXJ: resources, supervision, funding acquisition. DWM: conceptualization, resources, supervision, funding acquisition, project administration, writing—review and editing.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All procedures were in accordance with the ethical standards of Tianjin Central Hospital of Gynecology Obstetrics (Ethical approval number 2017KY009) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all the participants prior to participation in the study. All animal experiments were performed following the protocol approved by the Animal Ethical and Welfare Committee (AEWC) of Tianjin Medical University (Protocol number SYXK 2019-0004).

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Shiwen Xu, Xiaojing Guo or Weimin Deng.

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