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Simvastatin inhibits the immunosuppressive effects of endometrial cancer-associated mesenchymal stem cells through TGF- β 2/SMAD2/3 signaling and reduces tumor growth

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Simvastatin, a 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, was used in cardiovascular diseases and could decrease low-density lipoprotein cholesterol, and may have a repurposed role in cancer therapy. However, the effects of simvastatin on endometrial cancer remain controversial. We aimed to elucidate the role and mechanisms of simvastatin in regulating previously identified endometrial cancer-associated mesenchymal stem cells (EmCaMSCs)-mediated immunosuppressive effects and anti-tumor progression. Coculture of EmCaMSCs and peripheral blood mononuclear cells (PBMC) was used to assay the population of CD8+T cells, natural killer (NK) cells, and cytotoxicity of NK cells. The mechanisms were elucidated by applying recombinant proteins and inhibitors of candidate proteins, transforming growth factor-beta 2 (TGF- β 2). Finally, the humanized mouse model was generated to study the effects of simvastatin-mediated immunotherapy in treating endometrial cancer. The protein expressions of TGF- β 2, CD56, CD8, and PD-L1 in xenograft tumors were analyzed by Western blot or immunohistochemistry assay. In this study, simvastatin inhibited the proliferation of endometrial cancer cells (HEC-1 A and RL95-2) and EmCaMSCs, and the half-maximal inhibitory concentration (IC50) values of EmCaMSCs were much higher. Simvastatin rescued the proliferation and the population of CD8+T cells and natural killer (NK) cells from PBMC coculturing with EmCaMSC. Simvastatin treatment reduced the expression of TGF- β 2 in EmCaMSCs at both the gene and protein levels. TGF- β 2 activated the downstream SMAD2/3 signaling, and their inhibition by simvastatin could enhance the cytotoxicity of NK cells against endometrial cancer cells in vitro. Additionally, a combination of simvastatin and NK cell therapy inhibited xenograft growth, potentially by reducing TGF- β 2 expression. In conclusion, simvastatin could rescue the population of CD8+T cells and NK cells from PBMC cocultured with EmCaMSCs. Furthermore, simvastatin could enhance the cytotoxicity of NK cells in vitro and inhibit tumor growth in vivo in a humanized mouse model. These results suggested that simvastatin may be considered as a repurposed and combination drug for treating endometrial cancer.

Keywords Endometrial cancer, Cancer-associated mesenchymal stem cells, NK cells, Immunotherapy, TGF- β 2

Abbreviations

EmCaMSCs	Endometrial cancer mesenchymal stem cells
PBMC	Peripheral blood mononuclear cell

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NK	Natural killer
TGF- β	Transforming growth factor-beta
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
HMGCR	HMG-CoA reductase
LDL	Low-density lipoprotein
EMT	Epithelial-mesenchymal transition
TME	Tumor microenvironment
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1

Statins, traditionally applied in cardiovascular diseases to reduce cholesterol¹ and can be divided into two groups: type-I derivatives from fermentation, including mevastatin, lovastatin, pravastatin, and simvastatin, and type-II drugs from the synthetic origin, including fluvastatin, atorvastatin, cerivastatin, pitavastatin, and rosuvastatin². The primary role of statins in the mevalonate pathway is inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (HMGCR), resulting in the depletion of low-density lipoprotein (LDL) cholesterol³. However, recent studies suggested that statins exhibited anti-tumor effects, from meta-analysis and bench, in vitro and in vivo.

Statins could induce cancer cell apoptosis through the traditional caspase cascade and inhibit cell proliferation, migration, invasion, epithelial-mesenchymal transition (EMT), and chemoresistance in various types of cancer, including breast, lung, pancreas, and liver cancer⁴. Statins induced apoptosis of cancer cells through NF κ B and the canonical caspase pathway and reduced proliferation through MEK1/2, ERK1/2, and JNK pathways⁵. Statins also induced cell cycle arrest of cancer cells by activating AMPK and increasing p21 and p27 expression⁶. Simvastatin suppresses the invasion of cancer cells by decreasing Pituitary Tumor-Transforming Gene 1 (PTTG1)⁷. Furthermore, statins could also regulate epigenetic machinery, resulting in cell cycle arrest. DNA methyltransferase (DNMTs) could be the targets of statins, mediating the methylation of the downstream proteins, p16 and p21⁸. In conclusion, statins, especially simvastatin, showed anti-tumor progression in various cancers.

In several meta-analysis studies, statin use improved survival or reduced the risk of endometrial cancer^{9,10}. However, some studies did not support the protective effects of statins¹¹⁻¹⁵. Thus, the impact of statin use remains controversial in endometrial cancer patients. In the in vitro studies, statins exhibited anti-tumorigenic results in endometrial cancer cells when used alone⁹ or in combination with metformin¹⁶. These data suggested that statins could potentially be used for endometrial cancer. However, the detailed mechanisms are largely unknown.

On the other hand, statins may also affect the tumor microenvironment (TME), including immune cells and mesenchymal stem cells (MSCs). Statins mostly showed anti-inflammatory effects and enhanced the number of regulatory T cells (Treg)¹⁷ which obtained immunosuppressive effects on immunotherapy and may result in the suppression of the T helper 1 (Th1) immune response¹⁷. Besides, treatment of statins showed a reduction in the Th17 population¹⁸. On the contrary, a high dose of atorvastatin could reduce the in vitro function of conventional T cells and Treg¹⁹. Furthermore, statins were associated with better clinical outcomes in patients treated with PD-1 inhibitors²⁰. Statins plus Th1 cytokines or dendritic cells (DC)-based immunotherapy could suppress breast tumor growth²¹. Statins could stimulate immunogenicity, promote anti-melanoma immune response²² and enhance the responses to immune checkpoint blockade in non-small cell lung cancer (NSCLC)²³ and head and neck cancer cells²⁴.

Statins could enhance the osteogenic differentiation, angiogenic potential, migration, homing, survival, and proliferation of MSCs²⁵ which may have improved therapeutic outcomes in regenerative medicine. Simvastatin could decrease C-C motif chemokine ligand 3 (CCL3) expression from cancer cells and intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM1), interleukin-6 (IL-6), and CCL2 expression from cancer-associated MSCs (CaMSCs), disrupting the crosstalk of the cancer cells and TME and inhibiting tumor progression²⁶. The evidence of statins in regulating CaMSCs is limited.

MSC had a pleiotropic immunosuppressive function, including inhibition of NK cell proliferation, cytotoxicity and cytokine production, suppression of dendritic cell maturation and antigen presentation, and promotion of M2 polarization in macrophages²⁷. Besides, MSC suppressed B cell proliferation and immunoglobulin production, as well as T cell proliferation and effector function^{28,29}. Furthermore, MSC also regulated the expansion and function of myeloid-derived suppressor cells (MDSC) and differentiation of Treg³⁰. These immunosuppressive effects were mediated by IL-6, IL-10, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), and TGF- β from TME, including MSC or MSC-derived exosomes²⁷. Thus, MSC-mediated immunosuppression may contribute to tumor progression.

Simvastatin exhibits anti-metastatic and anti-tumorigenic effects in endometrial cancer cells through mitogen-activated protein kinase (MAPK)⁹. Simvastatin, combined with metformin, a drug for diabetes, synergistically inhibited cell growth and induced Bim expression and apoptosis in endometrial cancer cells through AMP-activated protein kinase (AMPK) signaling¹⁶. However, the effects of simvastatin in regulating endometrial cancer and TME are largely unknown due to the lack of in vitro and in vivo data.

TME is complex and essential for tumor progression. Among the cell components of TME, CaMSCs and immune cells play critical roles in tumor progression. Thus, we used endometrial cancer-associated MSCs (EmCaMSCs) cocultured with peripheral blood mononuclear cells (PBMC) and NK cells, and together with simvastatin to elucidate the roles and mechanisms of simvastatin in inhibiting the immunosuppressive effects of EmCaMSCs. These data may provide the foundation for the repurposing roles of simvastatin in treating endometrial cancer.

Materials and methods

Ethical declaration

The experimental procedures are approved by the Research Ethics Committee of Hualien Tzu Chi Hospital (IRB number: 109-257-A). This study adhered to relevant ethical standards and guidelines. Written informed consent was obtained from all participants.

Cell culture

Three primary EmCaMSCs (CaMSC1, CaMSC2, and CaMSC3) were obtained from our lab (Stem Cell Lab in Hualien Tzu Chi Hospital). The cells isolated as previously described²⁷ and cultured in low-glucose Dulbecco's Modified Eagle medium (DMEM, Sigma-Aldrich, St Louis, MO, USA), HEC-1 A were cultured in McCoy 5 A medium (Gibco, Waltham, MA, USA), and RL95-2 were cultured in DMEM/F12 medium (Gibco, Waltham, MA, USA). NK-92 cells were cultured in Myelocult H5100 medium (StemCell Technologies) supplemented with 12.5% horse serum (Gibco) and recombinant human IL-2 (500 U/mL, PeproTech, Cranbury, NJ, USA), which obtained better cytotoxicity³¹. Human peripheral blood mononuclear cells (PBMC) were donated from healthy persons and isolated using Ficoll-Paque (Amersham Biosciences, Piscataway, NJ, USA), following the manufacturer's instructions. PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco). The media were supplemented with 10% fetal bovine serum (FBS, Gibco) and 1× penicillin/streptomycin (PS, Gibco).

Growth inhibition assay

2×10^3 cells were seeded on a 96-well plate. After cell attachment, cells were treated with serially diluted simvastatin (Sigma-Aldrich) and cultured for 3 days. XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (Biological Industries Ltd., Beit Haemek, Israel) was used to measure the cell viability. IC₅₀ of simvastatin in each cell was calculated using GraphPad Prism software (version 9, GraphPad Software, Boston, MA, USA). Each experimental group was set up with three replicates, and the experiment was conducted three times independently.

Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR)

Total RNA was purified using the PureLink RNA Mini kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. According to the manufacturer's instructions, 1 μ g total RNA was reverse transcribed into cDNA using SuperScript III enzyme (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) and analyzed by Quant Studio 5 (Applied Biosystems). GAPDH was measured as an internal control, and all experiments were repeated in triplicate. The primer sequence is listed in Supplement Table 1.

Coculture system and LDH cytotoxicity assay

Briefly, PBMCs or NK-92 cells were cocultured with EmCaMSCs in the presence of IL-2 for 4 days. The ratio of PBMCs or NK-92 to EmCaMSCs was 10:1. For the cytotoxicity test in endometrial cancer cells, 1.5×10^4 RL95-2 and HEC-1 A cells were seeded and cultured overnight in a 96-well plate, respectively. After cell attachment, EmCaMSCs-cocultured PBMCs or NK-92 were harvested and further cocultured with cancer cells at a 10:1 ratio for 6 h in PBS containing 2% FBS and fresh IL-2. The supernatants were collected for the lactate dehydrogenase (LDH) activity test (ThermoFisher, Waltham, MA, USA) following the manufacturer's instructions. PBMCs or NK-92 cells cultured alone and treated with IL-2 were the control. Recombinant TGF- β 2 (3 ng/mL, PeproTech, Cranbury, NJ, USA) and anti-TGF- β 2 (300 ng/mL, Abcam, Cambridge, UK) were used in the experiments. All of the experiments were conducted in triplicate.

Flow cytometry analysis

For analysis of the immune cell population, the protein expression of CD56, CD8, and CD4 was analyzed. Briefly, PBMC cells alone or cocultured with EmCaMSCs were harvested and stained with anti-CD56-APC, anti-CD8-APC, and anti-CD4-PE antibodies (all from eBioscience, San Diego, CA, USA) for 30 min. For analysis of the signaling of the TGF- β 2/SMAD2/3 axis, NK-92 cells treated with recombinant TGF- β 2 and anti-TGF β 2 or simvastatin were harvested. Cells were fixed and stained with anti-TGF- β 2 (Abcam, Cambridge, UK), anti-SMAD2/3 (BD Biosciences, Franklin, NJ, USA), and pSMAD2/3 (Cell Signaling Technology, Danvers, MA, USA) in intracellular staining permeabilization wash buffer (BioLegend). After washing, cells were subsequently incubated with FITC-conjugated secondary antibody (Invitrogen). After the washout of the non-specific binding of antibodies, the target protein expression percentage was analyzed by flow cytometry (Lyric, BD Biosciences, Franklin, NJ, USA). Each experimental group was set up with three replicates, and the experiment was conducted three times independently.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of TGF- β 2 was determined using an ELISA Kit (ThermoFisher) according to the manufacturer's instructions. Briefly, the medium derived from CaMSCs or CaMSCs treated with simvastatin was incubated on an antibody-coated plate. After washing, the plate was further incubated with biotin-conjugated secondary antibody, streptavidin-horseradish peroxidase, and chromogen, respectively. The plate was analyzed using an ELISA reader (BioTek Synergy HTX, Agilent, Santa Clara, CA, USA) at 450 nm as the primary wavelength and 620 nm as the reference wavelength. Each experimental group was set up with duplicates, and the experiment was conducted three times independently.

Western blot

Cell or tissue lysates were collected, and proteins were purified using RIPA buffer (ThermoFisher) containing protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). Proteins were loaded into 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). After blocking with 1% BSA for 1 h, the proteins, including the phosphorylated form, were incubated with primary antibodies, targeting TGF- β 2 (Abcam, Cambridge, UK), pSMAD2/3 (SMAD2: Ser465/467; SMAD3: Ser423/425, Cell Signaling Technology, Danvers, MA, USA), SMAD2/3 (BD Biosciences, Franklin, NJ, USA), PD-L1 (Abcam, Cambridge, UK), and Actin (Cell Signaling Technology) at 4°C overnight and HRP-conjugated secondary antibodies (GeneTex, Irvine, CA, USA) for 1 h. The DAB substrate and ECL (ThermoFisher) were incubated with target proteins and detected by chemiluminescence. Each protein and its phosphorylated form were analyzed and quantified using ImageJ software (version 1.54i, NIH, Bethesda, MD, USA).

Animal study- humanized xenograft mouse model

The animal experiments were approved by the Animal Research and Care Committee of Hualien Tzu Chi Hospital (No. 110–28). The reporting of animal experimental protocols in the manuscript follows the recommendations in the ARRIVE guidelines. The animals were housed in standard cages with free access to water and food in a temperature-controlled environment under 12 12-hour light and 12-hour dark cycle. PBMCs were injected at a dose of 1×10^7 cells in an immunocompromised NOD-SCID mouse (strain name: NOD.CB17-Prkdcscid/Jtcu, purchased from Tzu Chi University) via the tail vein (IV) on day 0. Subsequently, 1×10^6 endometrial cancer cells and 1×10^5 EmCaMSCs (10:1) were subcutaneously (SC) co-inoculated into the left flank region on the same days. The mice were noted in the HEC-1 A group (control $n=5$, simvastatin, NK, and NK + simvastatin $n=4$ each) and in the RL95-2 group (control, simvastatin, NK, and NK + simvastatin, $n=4$ each). When the tumor grew to the optimal size, NK-92 cells (1×10^7 cells, IV injections, once) alone and simvastatin (1 mg/kg, PO, 5 times per week) alone, and the combinational treatment were administered. The control mice group received PBS, a replacement for simvastatin, and the tumor size was measured twice weekly. After the tumor size reached 1000 mm^3 , mice were sacrificed for tumor histopathological assays by CO_2 exposure, followed by decapitation. CO_2 gradually filled the euthanasia chamber, with the flow rate displacing 10–30% of the chamber volume per minute.

Immunohistochemistry analysis

The xenograft tumors were formalin-fixed, paraffin-embedded, and sectioned. The sections were first deparaffinized using xylene and then rehydrated. Antigen retrieval was performed by heating the sections in citrate buffer (pH 6.0) at 95 °C for 30 min. The sections were treated with 1% hydrogen peroxide and further blocked with 5% bovine serum albumin (Sigma), preventing nonspecific binding. Subsequently, the sections were incubated at 4 °C overnight with primary antibodies targeting TGF- β 2 (Abcam), CD56 (Cell Signaling Technology), CD8 (Cell Signaling Technology), and PD-L1 (Abcam) at a 1:100 dilution. The sections were then incubated with HRP-conjugated secondary antibody (GeneTex) for 1 h. Further, they detected the signal by using a DAB substrate kit (Dako, Carpinteria, CA, USA) at room temperature. The sections were counterstained with hematoxylin and photographed under a light microscope (Nikon TE2000-U, Tokyo, Japan).

Statistical methods

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 22, developed by the SPSS Institute in Chicago, IL, USA. Continuous variables were analyzed using the Student's t-test to compare the two groups. All values were expressed as mean \pm standard deviation (SD). Significance was set at $p < 0.05$.

Results

Statins could inhibit endometrial cancer cell proliferation

To determine the effect of simvastatin on cancer cells and EmCaMSCs, we evaluated the IC₅₀ of simvastatin on these cells. The IC₅₀ of simvastatin in HEC-1 A cells, RL95-2 cells, EmCaMSC2 (derived from a stage IA patient), and EmCaMSC3 (derived from a stage IIIC patient) were 2.5 μM , 2.8 μM , $\sim 50 \mu\text{M}$, and $\sim 10 \mu\text{M}$, respectively (Fig. 1). When taken together, simvastatin could inhibit endometrial cancer cell growth more than EmCaMSCs.

Simvastatin May increase the proliferation of PBMC by inhibiting EmCaMSC

To know the effect of simvastatin and EmCaMSC on the proliferation of PBMC, we added simvastatin and cocultured EmCaMSC with PBMC. PBMC (CD8, CD56, and CD4 population) reduced proliferation when cocultured with EmCaMSC. Adding simvastatin increased the PBMC proliferation in the cocultured condition (Fig. 2A-C). EmCaMSC-cocultured PBMC with cancer cells could decrease cancer cell death. After adding simvastatin, cancer cell death was increased (Fig. 2D).

Simvastatin decreased TGF- β 2 expression in CaMSCs

We initially screened several genes—including TNF- α , TGF- β 1, TGF- β 2, TGF- β 3, IL-8 (associated with cell proliferation), PCEAM (angiogenesis-related), EpCAM (involved in EMT and cell motility), LGR5 (a cancer stem cell marker), and PD-L1/PD-L2 (immune-related)—and identified TGF- β 2 as showing the most significant change in expression. To know the mechanism of simvastatin on cancer growth, we used Q-RT-PCR to evaluate TGF- β gene expression in EmCaMSCs and endometrial cancer cells. We aimed to assess the effects of clinically relevant concentrations of simvastatin (1 and 5 μM) on EmCaMSCs and cancer cells. TGF- β 1 influences the functions of MSCs across various aspects³². However, we found that, in EmCaMSCs, only TGF- β 2 was reduced

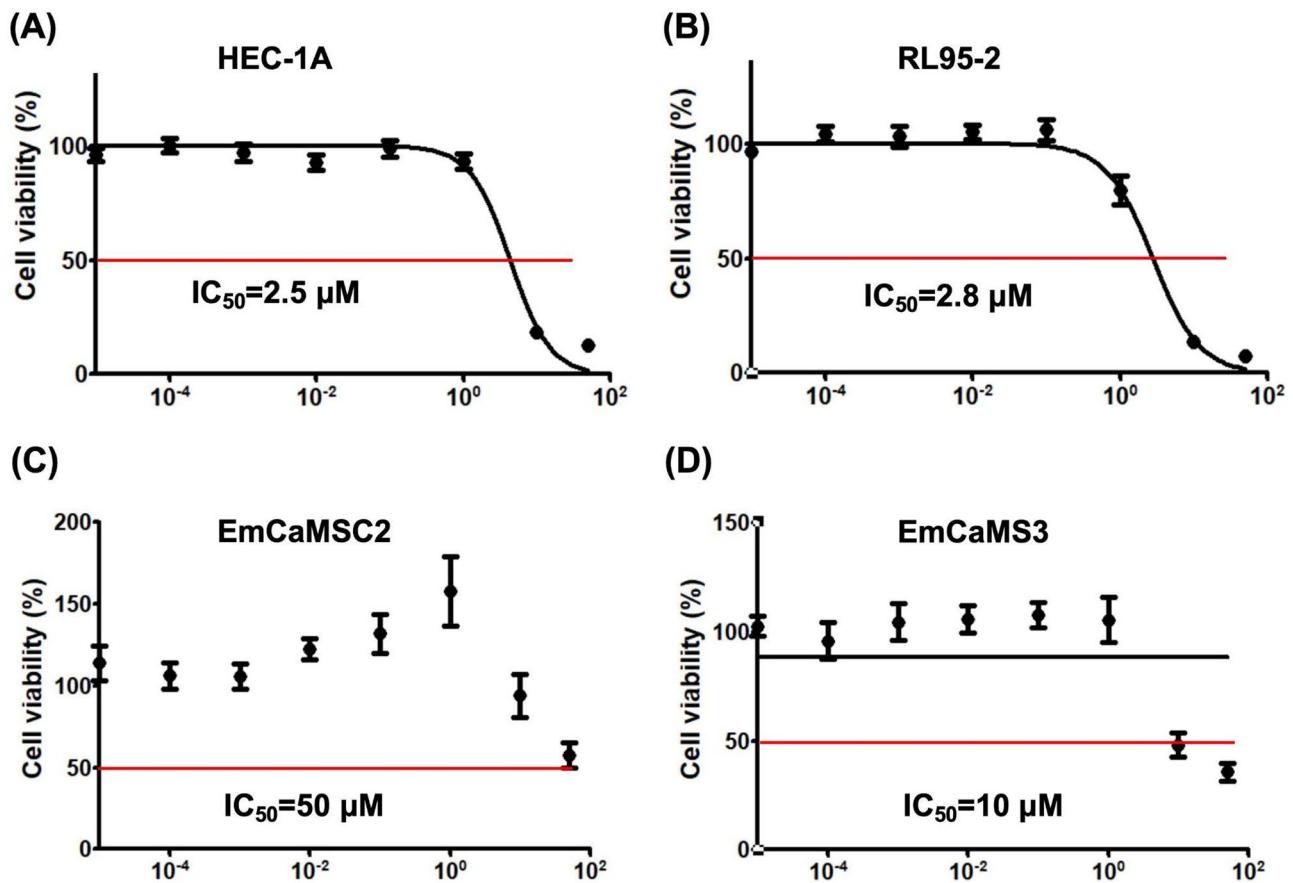


Fig. 1. Growth inhibition assay. Simvastatin was treated in different doses for 72 h in (A) HEC-1 A cells ($n=3$), (B) RL95-2 cells ($n=3$), (C) EmCaMSC2 (derived from stage IA patient) ($n=3$), and (D) EmCaMS3 (derived from stage IIIC patient) ($n=3$). The IC₅₀ of simvastatin in each cell from (A) to (D) was 2.5 μM , 2.8 μM , $\sim 50 \mu\text{M}$, and $\sim 10 \mu\text{M}$, respectively. Redline indicated 50% of inhibition.

after simvastatin 1 μM or 5 μM treatments (Fig. 3B), but not for TGF- $\beta 1$ or $\beta 3$ (Fig. 3A and C). Moreover, the expression of TGF- β did not alter in RL95-2 and HEC-1 A cells treated with simvastatin (Fig. 3D and F).

Next, we performed an ELISA to evaluate the protein secretion of TGF- $\beta 2$ in EmCaMSC and cancer cells after simvastatin treatment. Similar to the results of mRNA expression, the protein secretion of TGF- $\beta 2$ was also reduced after adding simvastatin, but not in cancer cells (Fig. 3G and H). In summary, simvastatin decreased 31.4% (from 22.2 to 11.8 pg/mL) of TGF- $\beta 2$ protein secretion from EmCaMSC. In summary, simvastatin specifically decreases TGF- $\beta 2$ gene and protein expressions in EmCaMSCs.

TGF- $\beta 2$ decreased PBMC proliferation by activating SMAD2/3

To evaluate the effect of TGF- β on the PBMC, we evaluated the proliferation and SMAD2/3 expression of PBMC. After adding recombinant TGF- $\beta 2$ protein, the sphere formation and proliferation of PBMC were significantly reduced (Fig. 4A-B). Phosphorylation of SMAD2/3 was increased (Fig. 4C). Given their well-characterized cytotoxic profile, NK-92 cells were used to further investigate the cytotoxic effects. NK-92 cells don't require antigen presentation via MHC to recognize and kill targets, while T cells rely on TCR-mediated and MHC-restricted antigen recognition. Adding TGF- $\beta 2$ could reduce the cytotoxicity of NK-92 cells in endometrial cancer cells, which could be reversed by treating TGF- $\beta 2$ antibody and simvastatin (Fig. 4D). Expression of TGF- $\beta 2$ and phosphorylation of SMAD2/3 in NK92 cells was increased when adding recombinant TGF- $\beta 2$ but further inhibited by applying anti-TGF- $\beta 2$ or simvastatin (Fig. 4E-F). We further validated the TGF- $\beta 2$ /SMAD2/3 signaling in NK-92 cells by flow cytometry analysis. Expression of intracellular TGF- $\beta 2$ remained unchanged in the control and treatment groups (Fig. 5A and B). Simvastatin treatment slightly decreased intracellular SMAD2/3 (Fig. 5A and C). Treatment of TGF- $\beta 2$ significantly increased the intracellular of pSMAD2/3 and decreased by cotreatment of anti-TGF- $\beta 2$ and simvastatin (Fig. 5A and D). Taken together, TGF- $\beta 2$ could reduce the cytotoxicity of NK-92 cells via activating SMAD2/3 in the tumor microenvironment. It could be inhibited by anti-TGF- $\beta 2$ or simvastatin.

Simvastatin and NK cells inhibited xenograft growth

Simvastatin enhanced NK cell cytotoxicity (as observed following anti-TGF- $\beta 2$ treatment, Fig. 4D) and reduced SMAD2/3 phosphorylation, mimicking the effects of TGF- $\beta 2$ blockade (Fig. 4E). Based on these findings, a

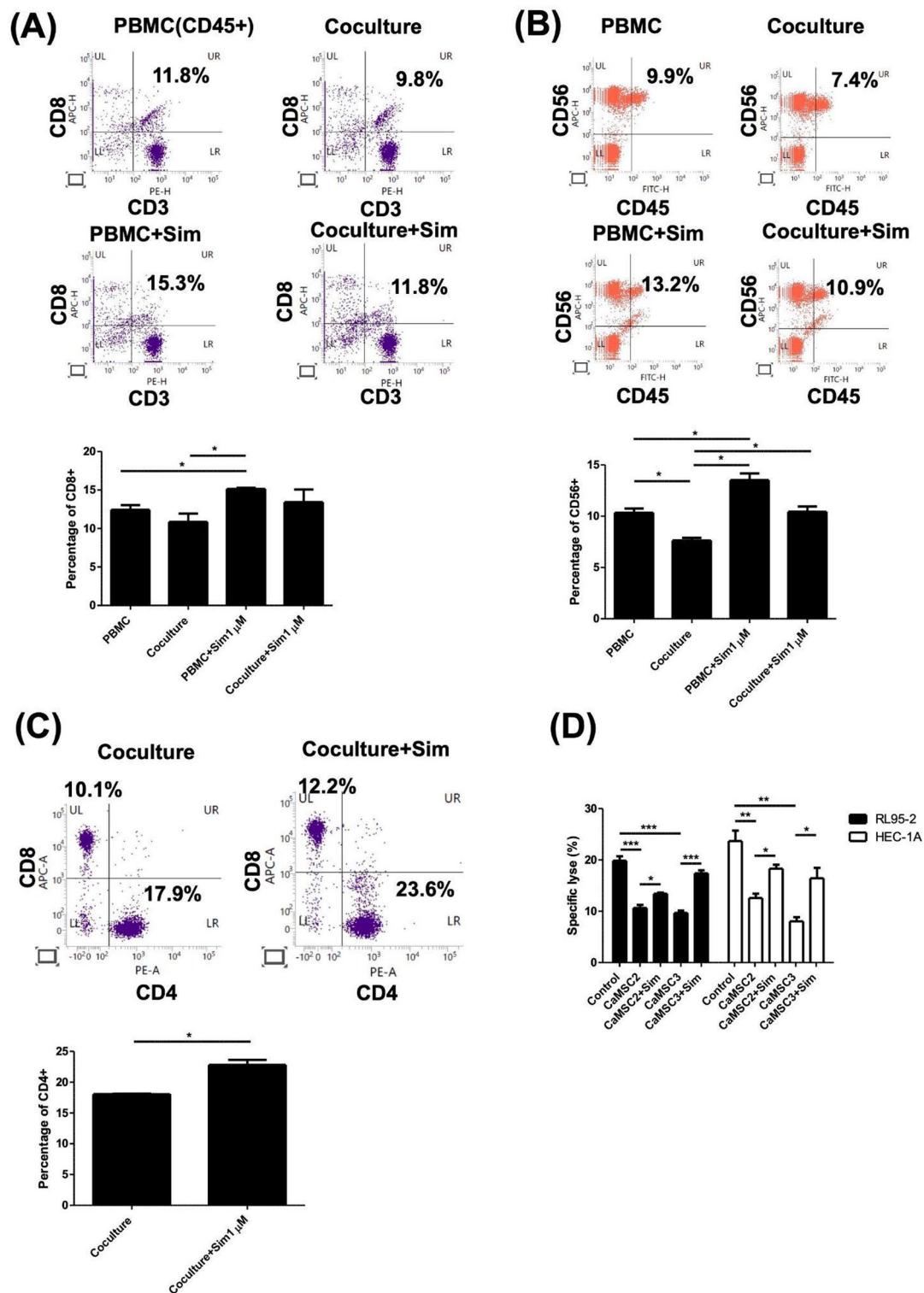


Fig. 2. The profile and cytotoxicity of PBMC cocultured with EmCaMSCs and simvastatin. PBMCs were cocultured with EmCaMSCs, simvastatin (1 μ M), and IL-2 (3,000 U/ml) for 4 days. Two EmCaMSCs (CaMSC2 and CaMSC3) were used for the cocultured experiments. Percentages of (A) CD8+ T cells ($n=3$), (B) CD56+ NK cells ($n=3$), and (C) CD4+ T cells ($n=3$) in PBMCs were analyzed by FACS. The representative flow pattern was shown on the upper panel. (D) Lactate dehydrogenase (LDH) cytotoxicity assay of PBMC cocultured with CaMSCs and simvastatin was calculated by measuring the LDH activity from the culture medium ($n=3$). PBMC alone was used for control. PBMC with lysis buffer was performed as a control (100% lysis), and those without lysis were taken as baseline. Sim: simvastatin. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

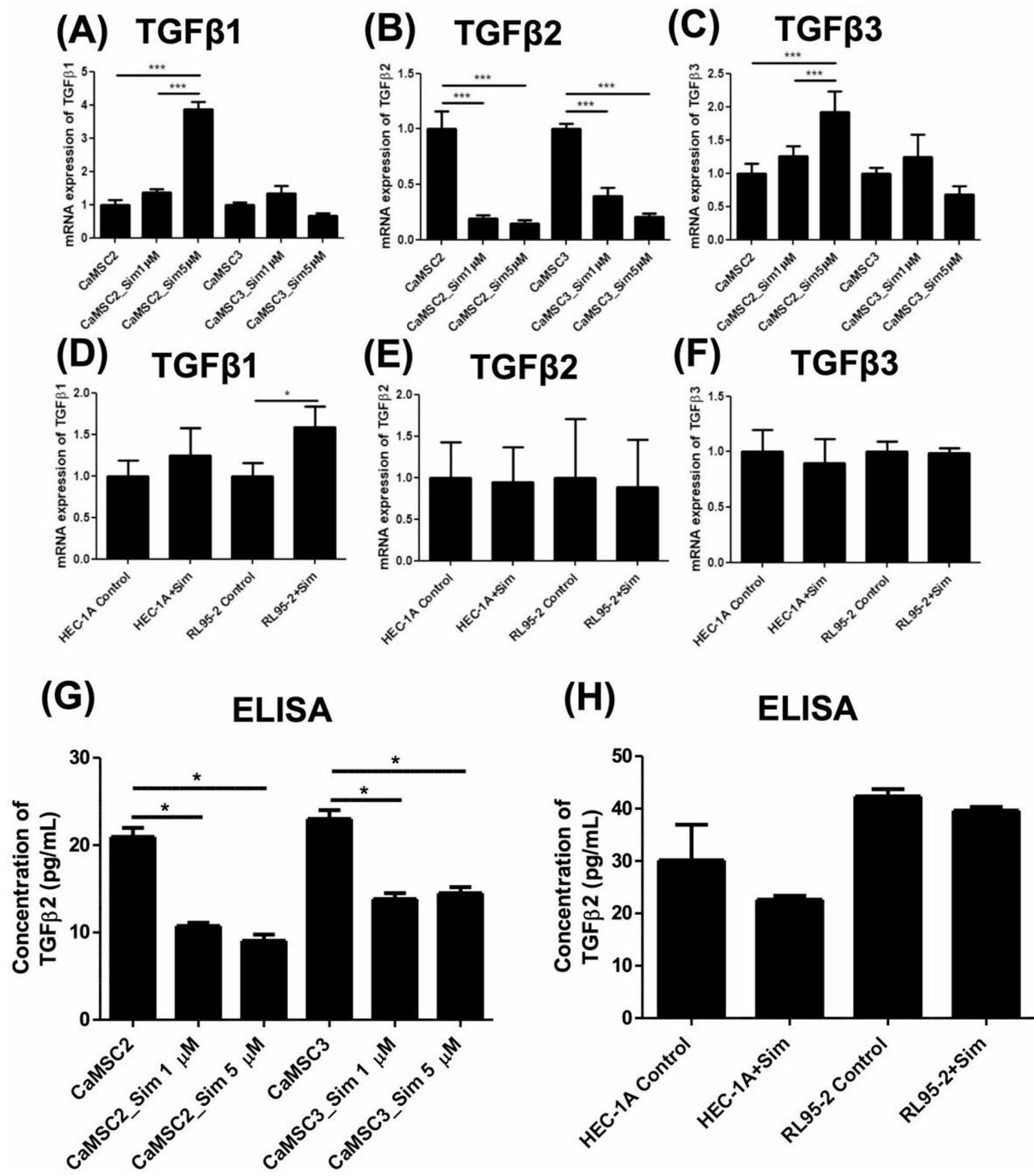


Fig. 3. Q-RT-PCR and ELISA analysis of $TGF\beta$ genes in EmCaMSCs and endometrial cancer cells. Simvastatin was treated in different doses for 24 h in CaMSC2, CaMSC3, RL95-2, and HEC-1A cells. mRNA expression of (A) (D) $TGF\beta$ 1 ($n=3$), (B) (E) $TGF\beta$ 2 ($n=3$), and (C) (F) $TGF\beta$ 3 ($n=3$), were analyzed. (G) ELISA of $TGF\beta$ 2 in CaMSC2 and 3 treated with simvastatin 1 and 5 μ M ($n=3$). (H) ELISA of $TGF\beta$ 2 in HEC1A and RL95-2 cells treated with simvastatin 1 μ M ($n=3$). Two concentrations (1 μ M and 5 μ M) of simvastatin were used in treating CaMSCs, and 1 μ M was used in treating endometrial cancer cells. Sim: Simvastatin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. GAPDH was used for endogenous control. ELISA: enzyme-linked immunosorbent assay.

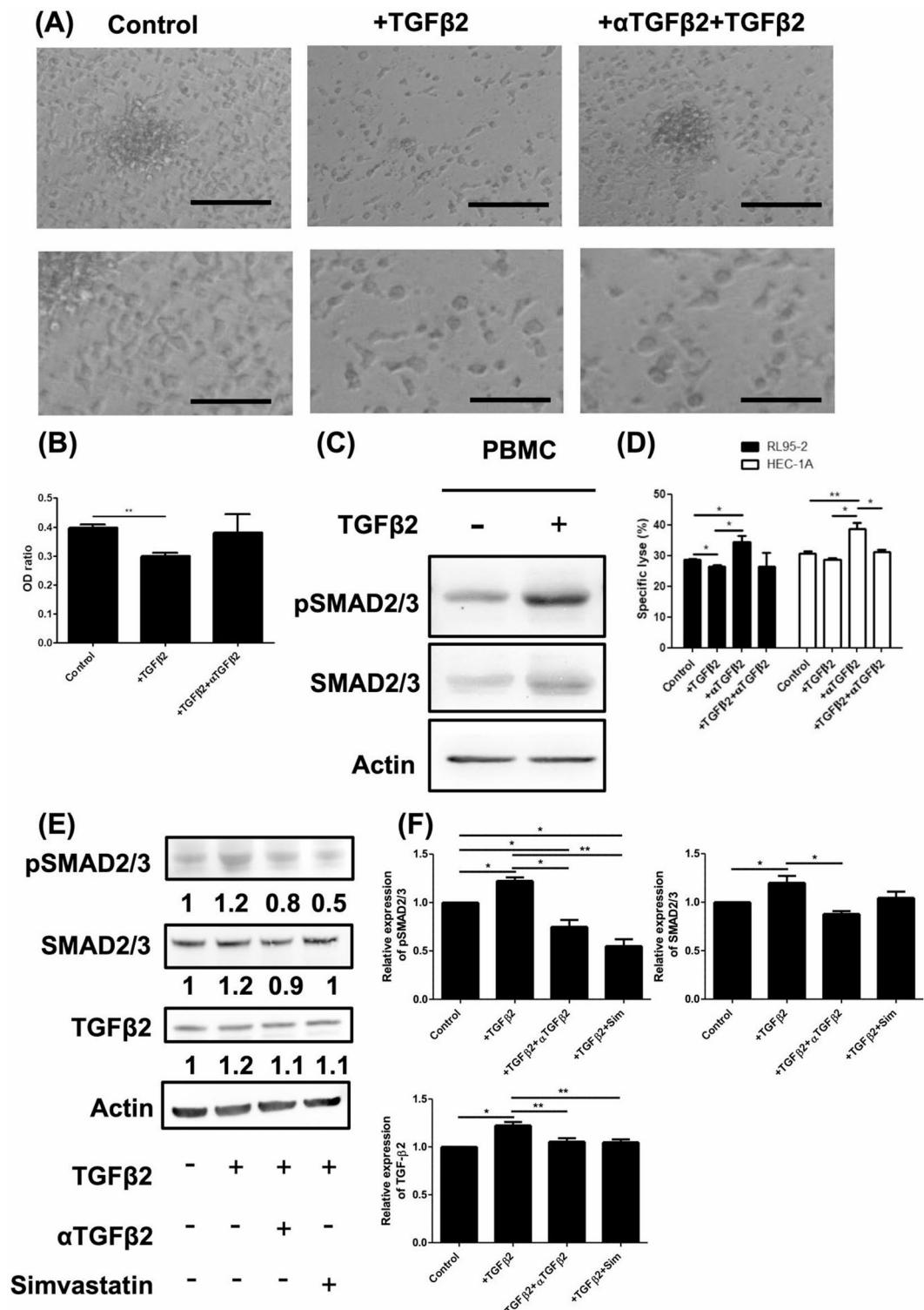


Fig. 4. The effects of TGF- β 2 on cytotoxicity. (A) The morphology (the bottom panel showed higher magnification than the upper panel) and (B) proliferation of PBMCs cultured with recombinant TGF- β 2 alone ($n=3$) and with anti-TGF- β 2 (α TGF- β 2) ($n=3$). (C) The downstream signaling of TGF- β 2 and SMAD2/3 in PBMC was analyzed by Western blot. (D) Cytotoxicity of NK92 cells against endometrial cancer cells ($n=3$). (E) The downstream signaling of TGF- β 2 and SMAD2/3 in NK92 cells was analyzed by Western blot. (F) Quantification of proteins listed in (E) ($n=3$). Scale bar: 100 μ m in the upper panel and 200 μ m in the lower panel. * $p < 0.05$, ** $p < 0.01$.

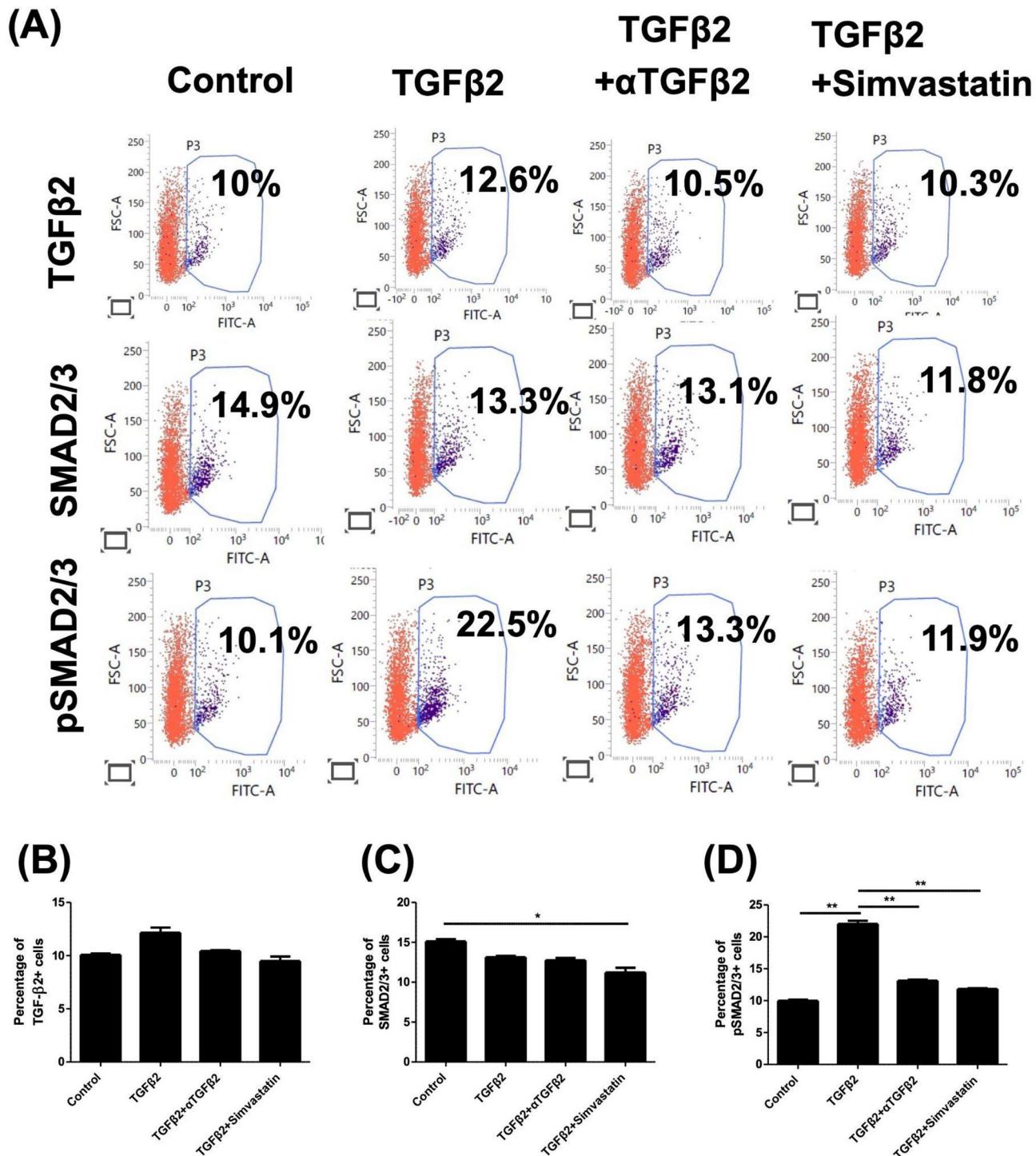


Fig. 5. The signaling of TGF β -2/SMAD2/3 in NK-92 cells by flow cytometry analysis. NK-92 cells were pretreated with anti-TGF- β 2 and simvastatin for 30 min and further treated with recombinant TGF- β 2 for 1 h. Untreated NK-92 cells were taken as control ($n=3$ for each group). (A) The percentage of intracellular TGF- β 2, SMAD2/3, and pSMAD2/3 was shown by flow cytometry. The quantitation of intracellular (B) TGF- β 2 ($n=3$), (C) SMAD2/3+ ($n=3$), and (D) pSMAD2/3+ cells ($n=3$) was shown. * $p < 0.05$, ** $p < 0.01$.

combination therapy using NK-92 cells and simvastatin was conducted. We used a humanized mouse model to determine the NK cells' and simvastatin's *in vivo* inhibitory effect on cancer growth. Human PBMC were first injected into the tail vein of NOD-SCID mice. Subsequently, the endometrial cancer cells (RL95-2 and HEC-1 A) and CaMSCs were injected into the subcutaneous region. After the tumor reached 150 mm^3 NK-92 cells alone, simvastatin alone, or in combination were given. We found that the NK and NK + simvastatin treatments could inhibit HEC-1 A + CaMSCs cell growth significantly ($p < 0.05$) than the control and simvastatin alone

(Fig. 6A-B). The tumor growth of the NK + simvastatin group was inhibited in RL95-2 + CaMSCs xenografts when compared with the control group (Fig. 6C-D). In summary, simvastatin, in combination with NK-92 cells, could reduce the tumor growth of endometrial cancer cells coinjected with CaMSCs.

TGF- β 2 and SMAD signaling were decreased in the xenograft tumors of simvastatin and NK cells treatment group
 To evaluate the mechanism of simvastatin and NK cell therapy in vivo, we surveyed the expression of TGF- β 2, SMAD2/3 signaling, and immune-related molecules programmed cell death 1 ligand 1 (PD-L1) by Western blot and the expression of TGF- β 2, CD56, CD8, and PD-L1 by immunohistochemistry analysis. The Western blot showed that TGF- β 2 and SMAD2/3 signaling were reduced in the NK + simvastatin group in the HEC-1 A + CaMSC xenograft tumor (Fig. 7A-B). Furthermore, the immunohistochemistry analysis showed that the expression of TGF- β 2 was dramatically decreased in the NK + Simvastatin tumors. NK cells (CD56+) were infiltrated into tumor lesions in NK alone and NK + Simvastatin tumors. The cytotoxic T cells (CD8+) were most abundant in the tumor margin in the control group and infiltrated into tumors in the treatment groups (Fig. 6C-D). These results also demonstrated that the humanized mice were successfully generated since the NOD-SCID mice are immunocompromised and devoid of T cells and NK cells. The expression of the immune checkpoint inhibitor, PD-L1, was slightly decreased in treatment groups (Fig. 7A-D). These data suggested that tumor growth inhibition may be associated with TGF- β 2-SMAD2/3 signaling and enhanced cytotoxicity of infiltrated NK cells (Fig. 8).

Discussion

Simvastatin reversed the immunosuppressive effects of EmCaMSCs, including increases of CD4+ and CD8+ T cells and NK cells and their cytotoxicity against endometrial cancer cells. In EmCaMSCs, TGF- β 2 was reduced after simvastatin treatment, but not TGF- β 1 and TGF- β 3. However, simvastatin treatment did not affect the expression of TGF- β s in endometrial cancer cells. TGF- β 2 and activation of SMAD2/3 signaling inhibited the cytotoxicity of NK92 cells and could be rescued by treating with anti-TGF- β 2 (α TGF- β 2) and simvastatin. Treatment of low-dose simvastatin (1 mg/kg) did not affect the xenograft tumor growth of coinjection of EmCaMSCs-endometrial cancer cells, and the combination of simvastatin treatment and NK cell therapy synergistically reduced the tumor growth in vivo. Expressions of TGF- β 2 and SMAD2/3 were downregulated in the simvastatin and NK cells combined treatment group. These results demonstrated that simvastatin could enhance the cytotoxicity of NK cells by inhibiting TGF- β 2/SMAD2/3 signaling in the TME.

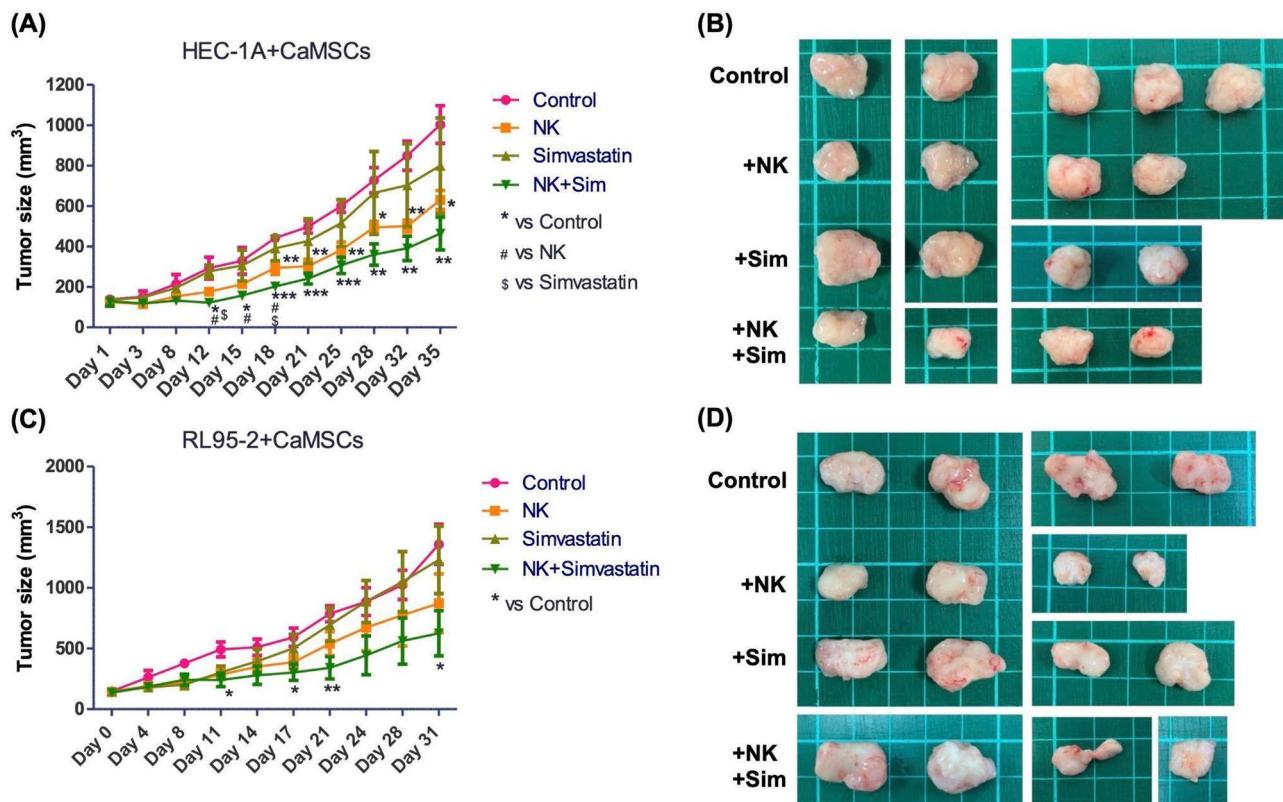


Fig. 6. Animal study- humanized mouse model. Tumor size was calculated by length x width x width/2 (mm^3). (A) HEC-1 A + CaMSCs (C) RL95-2 + CaMSCs. The mice were divided into (i) untreated ($n=5$) (HEC-1 A) or 4 (RL95-2)), (ii) treated with NK-92 cells ($n=4$), (iii) simvastatin (1 mg/kg) ($n=4$), and (iv) combination treatment with NK-92 cells and simvastatin ($n=4$). Gross pictures of xenograft tumors formed by (B) HEC-1 A + CaMSCs and (D) RL95-2 + CaMSCs in various groups. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

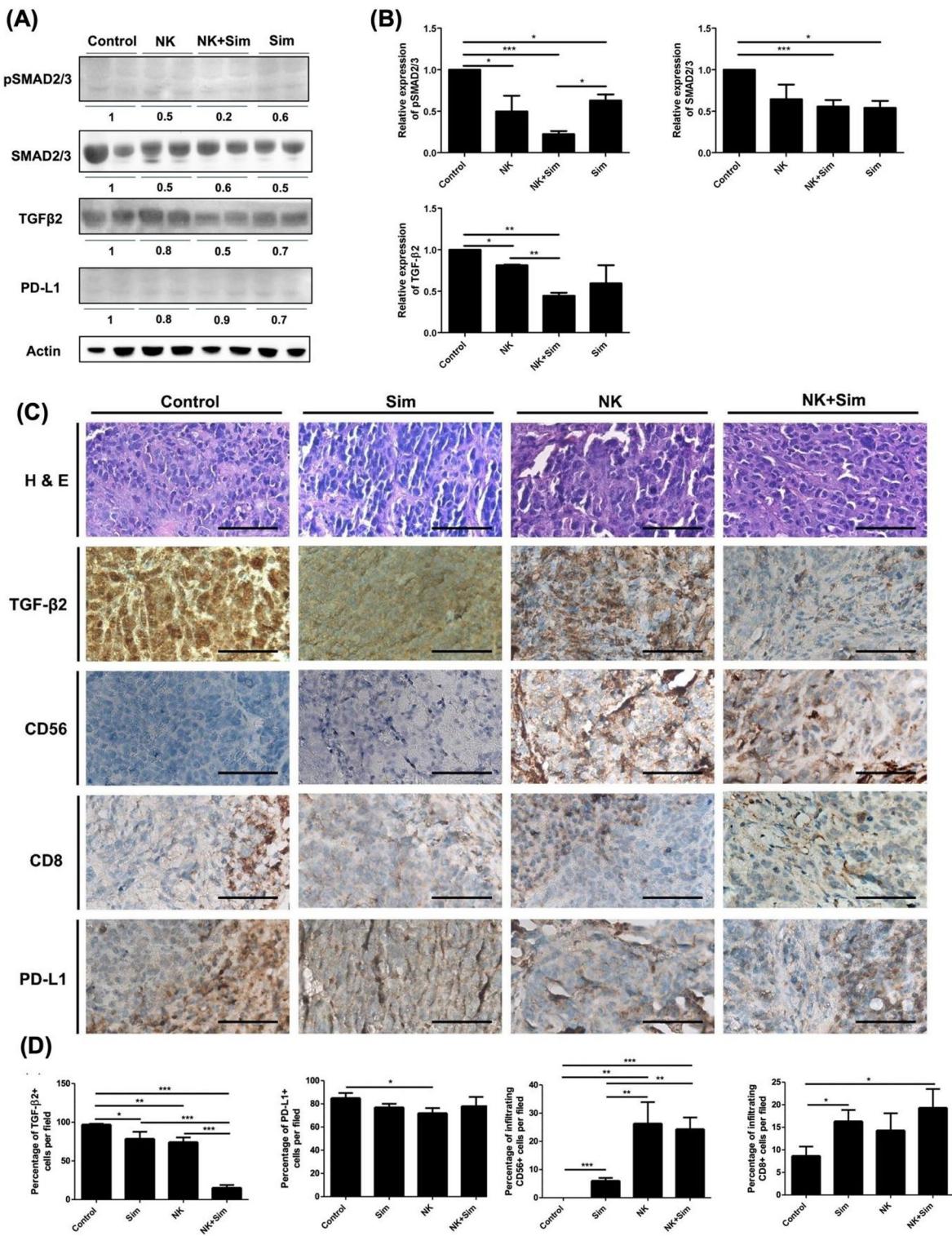


Fig. 7. Western blot and immunohistochemistry (IHC) analysis of xenograft tumors. **(A)** TGF- β 2, SMAD2/3 signaling, and PD-L1 in HEC-1 A + CaMSCs were analyzed by Western blot. **(B)** Quantification of proteins listed in **(A)** ($n = 3$). **(C)** The tumors derived from HEC-1 A + CaMSCs mice were formalin-fixed, paraffin-embedded, and sectioned. The samples were stained with TGF- β 2, CD56, CD8, and PD-L1. Hematoxylin and eosin (HE) confirmed cell morphology by staining the nucleus and cytoplasm. Scale bar: 100 μ m. **(D)** The quantitative results of the IHC ($n = 3$). The positive cells (in brown color) were counted from three fields in each group. Sim: simvastatin. NK + Sim: combination treatment with NK-92 cells and simvastatin. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

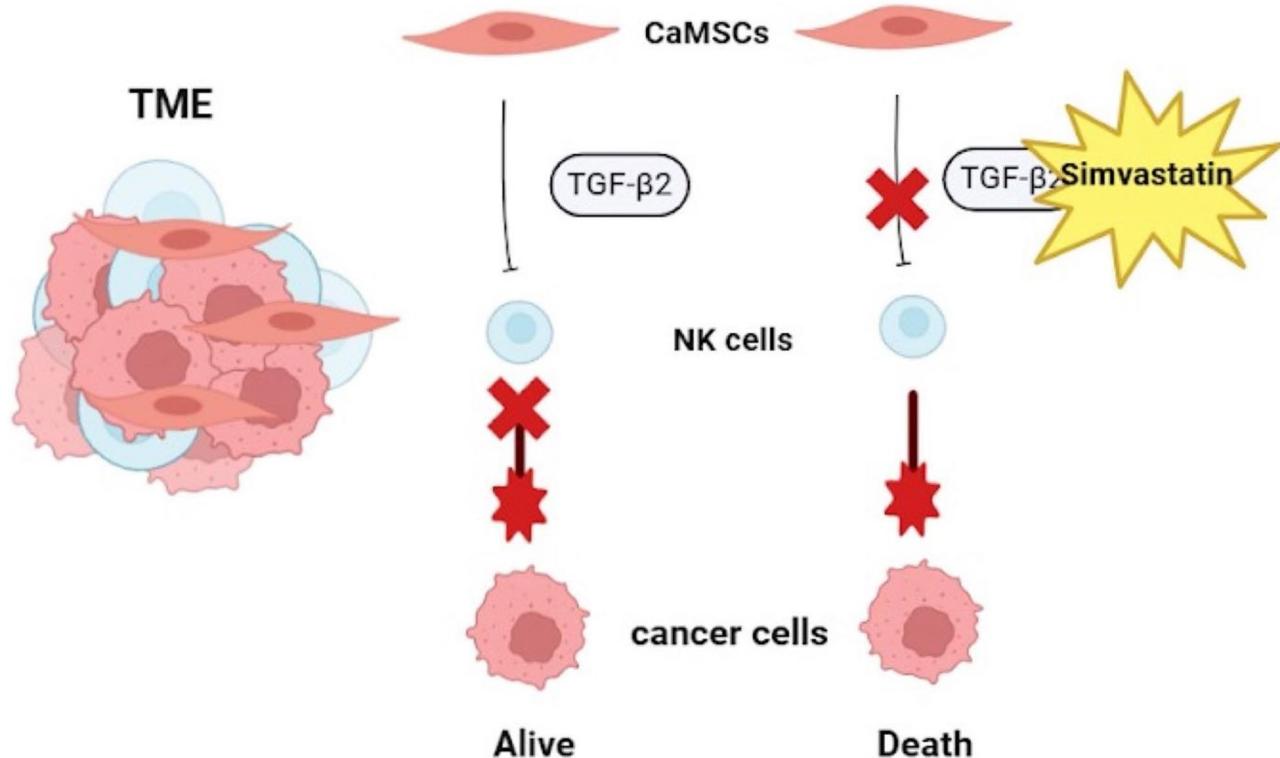


Fig. 8. The effects of simvastatin on the TME of endometrial cancer. By suppressing TGF- β 2, simvastatin inhibited the immunosuppressive effects of CaMSCs and enforced the cytotoxicity of NK cells against endometrial cancer cells.

The MSCs secreted immunomodulation molecules, including anti-inflammatory cytokines, TGF β 1, interleukin (IL)-13 IL-18 binding protein (IL18BP), ciliary neurotrophic factor (CNTF), neurotrophin 3 (NT-3) factor, IL-10, IL-12p70, IL-17E, IL-27, and IL1 receptor antagonist (IL1RA), and proinflammatory cytokines, IL-1b, IL-6, IL-8, and IL9³³. Among these molecules, the TGF β family and the downstream signaling have pleiotropic functions in regulating cancer cells as well as immune cells. TGF β signaling inhibited the proliferation and activation of NK cells and cytotoxic T cells³⁴. Thus, we surveyed the effects of simvastatin on the TGF- β signaling in the CaMSCs and cancer cells.

TGF- β signaling inhibited the activation and function of NK cells, resulting in anti-tumor immunity in solid cancer and B-acute lymphoblastic leukemia (B-ALL), which may be through the downstream SMAD pathway³⁵. Besides, TGF- β could repress mTOR (mammalian target of rapamycin) and further inhibit the activation of NK cells³⁶. NK cells from patients with metastatic breast cancer have reduced activation and function, and dysfunctional mitochondria, but increased expression of TGF- β and glycoprotein-A repetitions predominant (GARP)³⁷. Inhibiting TGF- β signaling by LY2157299 combined with adoptive NK cell therapy obtained better therapeutic efficacy than single treatment in the animal models of colon cancer³⁸. These results suggested that TGF- β , especially TGF- β 1, may inhibit NK function through different mechanisms in various cancers. Herein, we found that TGF- β 2 impaired the function of NK cells through SMAD2/3 signaling in the TME of endometrial cancer.

Studies have demonstrated that simvastatin inhibits the expression of TGF- β signaling in glioblastoma³⁹. This inhibition occurs through the suppression of the Rho/ROCK and Smad3 signaling pathway³⁹. In lung cancer cells, simvastatin downregulates TGF- β receptor II expression and inhibits cell proliferation via the ERK pathway⁴⁰. However, no study was noted regarding how statins affected cancer MSCs' TGF- β expression. In TME, neutralizing TGF- β enhances CD8+ T-cell and NK-cell-mediated anti-tumor immune responses^{41,42}. Inhibiting TGF- β signaling is an effective way to enhance the effector functions of NK cells in cancer. In this study, we discovered that simvastatin suppressed TGF- β 2-SMAD2/3 signaling within the TME, specifically affecting EmCaMSCs and NK cells.

Simvastatin induced ferroptosis of gastric cancer cells and activated anti-tumor immunity to sensitize anti-PD-1 immunotherapy⁴³. Both lovastatin and simvastatin enhanced T-cell killing of tumor cells when combined with PD-1 blockade in a head and neck cancer model²⁴. Statin users obtained improved overall survival (OS) and progression-free survival (PFS) than non-users in metastatic renal cell carcinoma patients receiving nivolumab (anti-PD-1)⁴⁴. Intriguing, post-immune checkpoint blockade (ICB) statin users showed significantly prolonged OS and PFS compared to pre-ICB statin and non-statin users⁴⁵. Bintralusp Alfa (BA), a bifunctional fusion protein targeting TGF- β and PD-L1, enhanced the survival in syngeneic ovarian cancer models by promoting T effector and NK cell responses⁴⁶. BA also advanced into clinical trials in treating various types of cancer, indicating

that TGF- β and PD-L1 may have synergistic effects on anti-tumor therapy. In this study, we demonstrated that simvastatin has the same potential in treating endometrial cancer. However, the effects of simvastatin combined with immunotherapeutic targets, such as immune checkpoint inhibitor PD-L1, need further investigation.

Simvastatin was found to reduce the expression of key immunosuppressive molecules—TGF- β 2—thereby rescuing immune cell populations and increasing NK cell cytotoxicity. The *in vivo* effects of simvastatin were confirmed using a humanized mouse model, where it inhibited tumor growth, likely through its immunomodulatory actions. Therefore, the effects of simvastatin combined with other drugs show potential efficacy in treating various types of cancer. For example, simvastatin and metformin, or combined anti-PD-L1, also showed therapeutic effects in many cancers^{47,48}.

There were some limitations in this study. The small sample size limited our study, as each group in the animal model included only four mice, with experiments repeated three times to ensure consistency. Additionally, we used two specific endometrial cancer cell lines, HEC-1 A and RL95-2, which may not fully represent the heterogeneity of endometrial cancer. These factors could limit the generalizability of our findings, and future studies with larger sample sizes and diverse cell lines or patient-derived xenografts are needed to validate our results. Furthermore, the variability in IC50 values between different cell types raises concerns about dose optimization, and the study does not address the long-term sustainability of simvastatin's effects. The syngeneic mice model of endometrial cancer needs to be generated to investigate the reactions of other immune cells when treated with simvastatin alone or in combination with other drugs.

Statins, including simvastatin, could decrease the PD-L1 expression in melanoma and lung cancer cells⁴⁹. Herein, we did not find decreases in PD-L1 in the xenograft tumors. We could not rule out that it was due to the low dose of simvastatin or that it was unique in endometrial cancer cells. Further studies were needed to clarify the role of simvastatin in regulating PD-L1 in endometrial cancer. Regardless of the role of PD-L1, simvastatin positively regulated the cytotoxicity of NK cells and reduced tumor growth, indicating that simvastatin may have the potential to enhance the efficacy of immune therapy.

For future research, we suggested investigating the interaction of simvastatin with other cancer treatment drugs (chemotherapy or targeted therapy) to explore potential synergistic effects. Additionally, we emphasized the need for further studies to validate the effects of simvastatin across a broader range of cancer types, using diverse cell lines, animal models, and clinical trials. These future investigations will help elucidate simvastatin's full therapeutic potential in oncology.

Conclusions

Simvastatin could rescue the population of CD8+ T cells and NK cells from PBMC cocultured with EmCaMSCs. Furthermore, simvastatin could enhance the cytotoxicity of NK cells *in vitro* and inhibit tumor growth *in vivo* in a humanized mouse model. These results suggested that simvastatin may be considered as a repurposed and combination drug for treating endometrial cancer. Further research is needed to fully understand its mechanisms, optimize dosing, and confirm its efficacy in more complex clinical settings.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

KHW conceived and designed the study. KHW performed the experiments. DCD, KHW, and YHC analyzed the data. DCD and KHW wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The human cell derivation and experiments were approved by the Research Ethics Committee of Hualien Tzu Chi Hospital (IRB number: 109-257-A).

Additional information

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