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RESEARCH

Combination therapy with expanded natural killer cells and atezolizumab exerts potent antitumor immunity in small cell lung cancer

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Abstract

Despite an initial response to platinum-based chemotherapy, most patients with extensive stage of small cell lung cancer (SCLC) have a poor prognosis due to recurrence. Additionally, the benefit of immune checkpoint inhibitors is more modest than non-small cell lung cancer. Natural killer (NK) cells can directly eliminate cancer cells without prior sensitization; this is largely governed by inflammatory cytokines, which serve as killing signals to cancer cells. Here, we investigated whether the combination of NK cells plus atezolizumab, a fully humanized monoclonal antibody that specifically targets the protein programmed death-ligand 1 (PD-L1), has a synergistic effect against SCLC. NK cells were expanded and activated using irradiated K562 feeder cells in the presence of interleukin (IL)-2/IL-15/IL-21/41BB ligand for 14 days. Expanded and activated NK cells (eNK) were combined with atezolizumab and used to treat SCLC cells in both in vitro and in vivo studies. The results revealed increased PD-L1 expression in SCLC cells after the eNK challenge. eNK cells plus atezolizumab demonstrated increased cytotoxicity toward target SCLC cells, as evidenced by increased interferon-γ and tumor necrosis factor-α production, and higher levels of SCLC stem cell ($CD44^{+}CD90^{+}$) suppression. Combined treatment with eNK and atezolizumab more effectively inhibited SCLC tumor growth and significantly prolonged the survival of treated mice. Our findings revealed that combining eNK with atezolizumab strongly increased cytotoxicity, significantly inhibited SCLC tumor growth, and prolonged the survival of treated mice. These results provide a framework for developing a more advanced immunotherapeutic modality for future clinical trials for patients with SCLC.

Keywords Small cell lung cancer · NK cells · Anti-PD-L1 · Atezolizumab · Combination therapy

Introduction

Small cell lung cancer (SCLC) accounts for approximately 10–15% of all lung cancers and is characterized by rapid growth and metastasis with poor prognosis [1–3]. Recently, atezolizumab or durvalumab along with platinum-etoposide chemotherapy has been considered as the new standard of care in the first-line setting in extensive stage SCLC (ES-SCLC) [2, 4]. Immune evasion mechanisms are critical survival strategies for cancer cells to avoid recognition and destruction by the immune system [5]. Binding between programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) is a critical mechanism by which cancer cells can evade the host immune response and provide immune evasion [6]. However, in SCLC, the magnitude of benefit with this combination strategy is more modest than that observed in patients with metastatic non-small-cell lung

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cancer (NSCLC) because of the immunosuppressive features of SCLC [2]. SCLC is characterized by inherently low immunogenicity, contributing to minimal immune cell infiltration. This "immune desert" phenotype arises from several factors, including downregulated major histocompatibility complex (MHC) expression, impaired antigen presentation, the presence of high levels of immunosuppressive cells, and limited expression of immune checkpoint molecules such as PD-L1. Moreover, the presence of a cancer stem cell (CSC)-enriched subpopulation, which may underlie the tumor's drug resistance [3, 7]. In the context of SCLC, significant advancements in immune checkpoint inhibitor (ICI)-based treatments were not achieved until 2019, with the approval of two PD-L1 inhibitors, atezolizumab and durvalumab. These approvals were based on the survival benefits demonstrated in the IMpower133 [8] and CASPIAN [9] clinical trials, where ICIs were combined with chemotherapy. However, the therapeutic response to ICIs in SCLC has proven to be neither consistent nor durable, with survival benefits becoming evident only after approximately six months of treatment. These challenges underscore the need for a deeper understanding of the mechanisms limiting ICI effectiveness in SCLC and the development of strategies to overcome these barriers.

Natural killer (NK) cells play a crucial role in immune surveillance against cancer, and their activity is regulated by a complex interplay of activating and inhibitory signals [10]. NK cells have been shown to recognize and kill SCLC cells [11, 12], while several preclinical and clinical studies have investigated the use of NK cells in SCLC immunotherapy [13, 14]. In some cases, the use of NK cells has been associated with improved response rates and survival; however, the activity of NK cells is often suppressed in the tumor microenvironment (TME), which allows cancer cells to escape and progress [12–14]. Understanding the mechanisms by which the TME suppresses NK cell activity is important for developing new immunotherapies that can overcome these barriers and enhance the immune response against cancer [12].

Numerous studies have shown that activating signals from T cells, NK cells, and other immune cells, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, can induce PD-L1 expression in cancer cells [12, 15]. PD-L1 on cancer cells delivers a suppressive signal to immune cells and an antiapoptotic signal to cancer cells [12, 15], serving as a protective mechanism that inhibits immune cell activity, allowing cancer cells to survive and grow. Cancer cells produce various immunosuppressive factors, including transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), and interleukin-10 (IL-10). These factors work together within the TME to establish a strongly immunosuppressive and pro-tumorigenic setting. These inhibitory factors can also induce the expression of PD-1 on T cells, NK cells, and other immune cells [12, 15, 16]. Previous studies have demonstrated that PD-L1 is not only expressed on cancer cells but also on immune cells, such as NK cells and macrophages, within the TME [17, 18]. However, it remains unclear whether anti-PD-L1 blockade can improve the function of PD-L1-expressing NK cells.

In the present study, we investigated whether the combination of expanded and activated NK cells (eNK) and atezolizumab, a monoclonal antibody (mAb) to PD-L1, has a synergistic effect on SCLC cell lines, with the aim to assess the potential for improved antitumor efficacy of standard chemoimmunotherapy in ES-SCLC.

Materials and methods

Ethics declaration

All experimental procedures were approved by the Institutional Review Board of Chonnam National University Hospital. All animal experiments were performed in accordance with protocols approved by the Chonnam National University Animal Use and Care Committee.

Cell lines and atezolizumab

Human small cell lung cancer cell lines, including SHP77 (Cat #: CRL-2195), H69 (Cat #: HTB-119), and H209 (Cat #: HTB-172), were purchased from the American Type Culture Center (ATCC, Manassas, VA, USA). Human SCLC cell lines DMS273 (Cat # 95,062,830-1VL) were obtained from Sigma Aldrich. SBC-5 cells were cultured in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin, and SHP77, DMS273, H69, and H209 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified 5% CO_2 incubator. Anti-PD-L1 mAb (atezolizumab) was purchased from Selleck Chemical Company (Texas, USA).

Ex vivo NK cell activation and expansion

NK cells were expanded and activated using our recently established K562 feeder cells. Briefly, peripheral blood mononuclear cells isolated from healthy donors were co-cultured with gamma-irradiated (100 Gy) K562 feeder cells in RPMI-1640 medium containing 10% FBS, 1% penicillin/streptomycin, and 4 mM L-glutamine in the presence of 10 U/mL recombinant human IL-2 (Peprotech, Rocky Hill, NJ, USA), 5 ng/mL IL-21, and 10 ng/mL 41BB ligand. On day 3 of culture, the medium was changed in the presence of 10 U/mL IL-2, and both the medium and cytokine were replaced every 2–3 days. After 7 days of culture, the concentration of

IL-2 was increased to 100 U/mL, and 10 U/mL of recombinant human IL-15 (Peprotech) was added to the medium. On day 14 of culture, eNK cells were harvested and used for in vitro and in vivo experiments.

Immunofluorescence analysis by flow cytometry

The purity of eNK cells was analyzed by flow cytometry after staining with fluorochrome-conjugated monoclonal antibodies (mAbs) (CD3 and CD56). Briefly, eNK cells (2×10^5 cells) were stained with appropriate antibodies for 15–20 min, before analyzing on a BD FACSCalibur flow cytometer. Intracellular levels of IFN-γ, granzyme-B (GRZB), FasL, and Ki67 in eNK cells were measured using Fixation/Permeabilization Solution 2 (eBioscience). Flow cytometry data were analyzed using FlowJo software.

Analysis of PD-1 and PD-L1 expression on the surface of eNK and SCLC cells

SCLC cell lines (SBC-5, SHP77, DMS273, H69, and H209) were transduced with GFP-lentivirus, and SCLC-GFP-positive cells were isolated using fluorescence activated cell sorting (FACS). Subsequently, GFP-positive SCLC cell lines were co-cultured with eNK cells, and the levels of PD-1 and PD-L1 on cancer cells and eNK cells were analyzed using a FACscanto II flow cytometer. Flow cytometry data were analyzed using FlowJo software.

Examination of cancer stem cells and non-neuroendocrine/mesenchymal-like phenotypes in SCLC

SCLC cell lines (SBC-5, SHP77, DMS273, H69, and H209) were co-cultured with eNK cells, and the levels of stem cell SCLC and non-neuroendocrine/mesenchymal-like SCLC phenotypes were analyzed on a FACscanto II. Flow cytometry data were analyzed using FlowJo software.

Lactate dehydrogenase (LDH) release cytotoxic assay

The quantitative cytotoxic assay for measuring LDH was performed as described previously [19]. We used the CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA) to analyze the direct killing effect of activated lymphocytes against SCLC cell lines, according to the manufacturer's instructions. SCLC cell lines were co-cultured with eNK cells at a 1:1 ratio in the presence or absence of 10 μg/mL of atezolizumab in a Costar 96-well plate (Corning, USA) for 6 h at 37 °C with 5% CO_2. Subsequently, the supernatants were collected to measure the concentration of LDH, which is a cytosolic enzyme released upon cell lysis. The percentage of cell lysis was calculated according to the manufacturer's instructions.

CFSE-based cytotoxicity assay

The cytotoxicity of eNK cells against SCLC target cells was assessed using a CFSE-based assay. Briefly, target cells were stained with 0.5 μM CFSE in FACS buffer for 10 min at 37 °C, washed twice with complete media, and then 5×10^4 target cells were plated in triplicate in a 96-well U-bottom plate. The target cells were then mixed with eNKs at a 1:1 effector-to-target (E:T) ratio. The plates were centrifuged at 1,500 rpm for 3 min and incubated for 4 h at 37 °C in a 5% CO_2 incubator. After incubation, the mixed cells were transferred to FACS tubes, and 1 μL of 1 mg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) was added to each tube before acquisition. Cells were acquired on a BD FACS Canto II (Becton Dickinson), and the data were analyzed using FlowJo software (TreeStar). The percentage of dead target cells (CFSE-positive and PI-positive) was calculated after subtracting the percentage of spontaneous death of target cells.

CD107a degranulation and intracellular cytokine staining assay

To assess the effects of atezolizumab on the ability of NK cells to degranulate SCLC cells, we co-cultured SCLC cell lines (SBC-5, SHP77, DMS273, H69, and H209) with eNK cells (E:T of 1:1) in the presence of a PE-conjugated anti-CD107a antibody in 96-well U-bottom plates for 1 h. Brefeldin A and monensin (BD Biosciences) were added, and the cells were incubated for an additional 3 h at 37 °C in a 5% CO_2 incubator. After incubation, the cells were stained for hCD45 for 30 min. After washing, the cells were permeabilized using FACSTM Permeabilizing Solution 2 (BD Bioscience) at room temperature for 30 min. The cells were then stained with intracellular antibodies, including IFNγ, Granzyme B (GRZB), Ki67, and FasL for another 30 min at room temperature. The samples were analyzed on the BD FACS Canto II, and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

SCLC xenograft model

NOD/SCID IL-2Rγnull (NSG) mice were purchased from the Jackson Laboratory (Bar Harbor, MA, USA). To establish an SCLC xenograft model, we subcutaneously injected human DMS273 cells into 9–12-week-old male and female mice. The following five treatment groups (seven mice per group) were established: (1) phosphate buffered saline (PBS) control, (2) PD-L1 blockade, (3) eNK, and (4) eNK + PD-L1 blockade. On day 0, mice were subcutaneously injected in

the right flank with 1×10^7 DMS273 cells in a volume of 0.1 mL. After tumor growth, freshly harvested eNK cells (1×10^7 cells/mouse) were infused once per day for 9 days in three cycles (1 week one cycle, with 3 days of eNK infusion) on days 14–16, 21–23, and 27–29. The PD-L1 blockade agent (200 μg/mouse) was injected intraperitoneally in a volume of 0.1 mL on days 15, 22, and 28. To assess the antitumor status of treated mice, we measured the length, width, and height of each tumor every 3 to 4 days using a Vernier caliper, and the tumor volume was calculated using the standard formula for calculating the volume of an ellipsoid: $V = 4/3\pi$ (length × width × height/8). Serum pro-gastrin-releasing peptide (proGRP) levels were assessed using enzyme-linked immunosorbent (ELISA) assay. In vivo persistence and tumor infiltration of eNK cells were assessed by flow cytometry. Mice were euthanized when the tumor reached 2,000 mm^3, which was considered equivalent to death due to the large size of the tumor.

Statistical analysis

Data were analyzed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was determined using two-way analysis of variance (ANOVA) of variance. *P*-values < 0.05 were considered significant. Data are expressed as the mean ± standard deviation or standard error of the mean.

Results

Increased expressions of PD-L1 and PD-1 on the surface of eNK and SCLC cells after co-culture

Because the expression level of PD-L1 is very low on SCLC (< 30%), resulting in a limited response to PD-L1 blockade, we first focused on the effects of eNK cells for the possibility of inducing PD-L1 expression on the SCLC cell surface. After expansion and activation of NK cells for day 14 of culture, eNK cells showed high purity (> 90%) and high expression of various activating surface receptors, including LFA1, NKG2D, NKP44, and NKP46 (Supplementary Figs. 1A–E). In this study, various SCLC cell lines, including SBC-5, SHP77, DMS273, H69, and H209, were transduced by GFP lentivirus, and GFP-positive SCLC cells were sorted by FACS. GFP-positive SCLC cell lines were co-cultured with eNK cells, and the percentages of PD-L1 expression on SCLC cell lines were evaluated. The results showed dramatically increased expression of PD-L1 on the surface of SCLC cell lines: the levels of PD-L1 on SCLC cell lines were approximately 30% after 6 h co-culture (Fig. 1A and B) and nearly 85% following overnight co-culture (Fig. 1C and D) The killing activities of eNK cells, as measured by IFN-γ secretion, are likely to play a critical role in the induction of PD-L1 expression in SCLC cell lines (Supplementary Fig. 1F). In addition, eNK cells showed a dramatic increase in PD-L1 expression after co-culture with SCLC cell lines for 6 h (Fig. 1E and F) and overnight (Fig. 1E and G). This suggests the increased upregulations of PD-L1 molecules on eNK and SCLC cells may be acquired via contacting, which SCLC cells may provide the PD-L1 molecules to eNK cells. Additionally, eNK cells cultured in transwell with SCLC cells (where physical interaction between the eNK and SCLC cells is precluded) failed to stain PD-L1 (Fig. 1H and I). This further supports the conclusion that direct cell–cell contact is essential for PD-L1 upregulation on eNK cells.

We further evaluated the change in PD-1 expression in SCLC cell lines and eNK cells after co-culture. Upregulation of PD-1 in cancer cells is associated with immunosuppressive factors secreted in the TME, which lead to the suppression of the immune response against cancer [7, 15]. SCLC cells showed dramatically increased PD-1 expression after co-culture with eNK cells for 6 h (Fig. 2A and B) and overnight (Fig. 2C and D). eNK cells also exhibited upregulation of PD-1 expression after co-culture with SCLC cells for 6 h (Fig. 2E and F) and overnight (Fig. 2E and G). Notably, eNK cells cultured in transwells with SCLC cells partly increased the expression of PD-1 molecules (Fig. 2H and I). These results showed that co-culturing eNK cells with SCLC cell lines modulated the cellular abundance of PD-L1 expression on SCLC cell lines, and induced high levels of PD-1 expression on both eNK cells and SCLC cell lines.

Furthermore, we co-cultured SCLC cell lines with eNK cells overnight, with or without PD-L1 blockade. The addition of PD-L1 blockade resulted in a partly decrease in the expression of suppressive molecules such as CD73, CTLA4, TIM3, and LAG3 on eNK cells (Supplementary Figs. 1G– J). CTLA-4 is well-known in inhibiting CD28-mediated costimulation, a critical pathway in T cell activation and an essential regulator of immune responses. Beyond T cells, the CD28 pathway also plays a role in NK cell cytokine secretion and proliferation, as evidenced by reduced tumor-lysing capacity in CD28-deficient NK cells [20, 21]. Treatment with ipilimumab, an anti-CTLA-4 antibody, depletes regulatory T cells and enhances NK cell function in advanced melanoma patients [22]. LAG-3 ligands, including LSECtin and galectin-3, are tumor-expressed inhibitors of antitumoral immunity [23]. Galectin-3 is also present in resting human NK cells, with expression modulated by cytokines [24]. TIM3 mediates immune exhaustion in T cells, contributing to impaired responses in chronic infections and tumors. Blocking Tim-3 reverses exhaustion in $CD4^+$ and $CD8^+$ T cells in conditions like melanoma. Interestingly, NK cells constitutively express TIM-3, and its blockade restores their function, correlating with disease stage and prognosis [25].

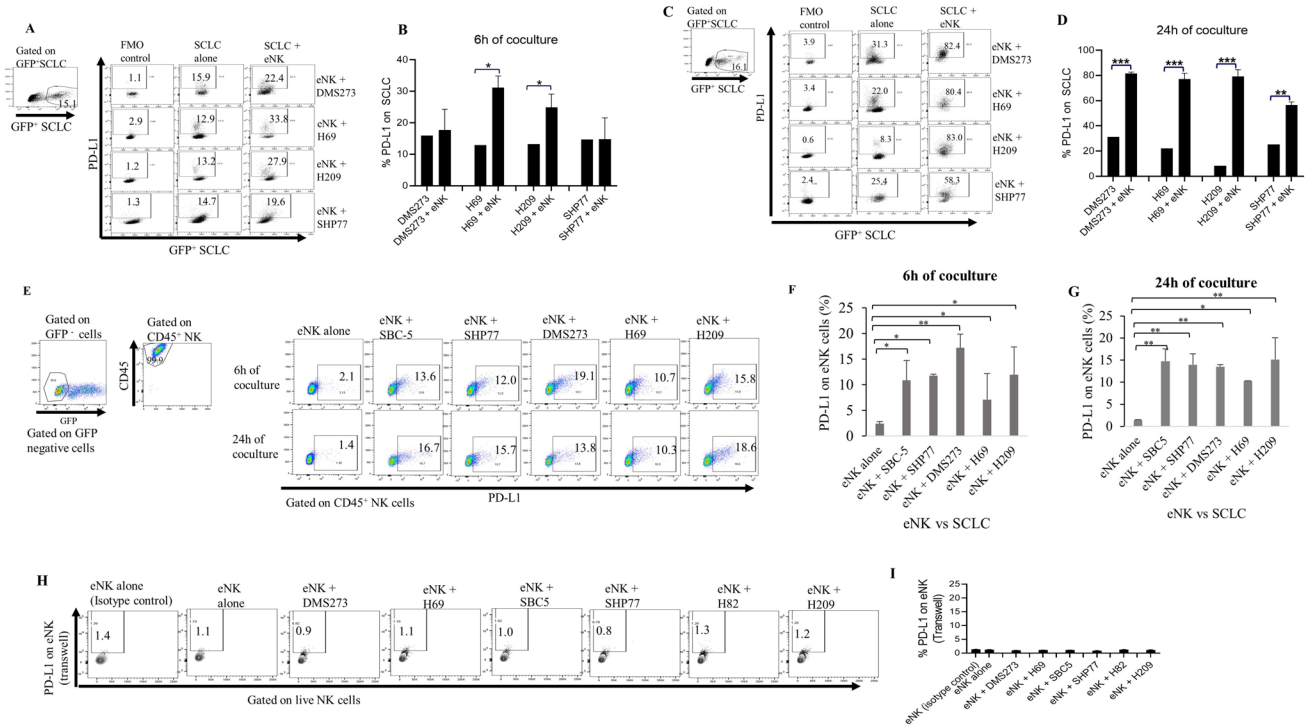

Fig. 1 Significant upregulation of PD-L1 molecules on the surface of SCLC and eNK cells after coculture. GFP-positive SCLC cell lines, including SBC-5, SHP77, DMS273, H69, and H209, were co-cultured with eNK cells to evaluate PD-L1 expression levels on both SCLC and eNK cells. PD-L1 expression on SCLC cells was assessed at 6 h of co-culture (**A** and **B**) and following overnight co-culture (**C** and **D**) using flow cytometry. Similarly, PD-L1 expression on eNK cells after co-culture with SCLC cell lines was measured at 6 h (**E** and **F**) and overnight (**E** and **G**) using flow cytometry. Differences between groups were analyzed using two-way ANOVA. (* $P<0.05$, ** $P<0.012$, *** $P<0.001$). (**H** and **I**) PD-L1 expression on eNK cells after cultured in transwell with SCLC cells. Data are representative from three independent experiments (n = 3)

Additionally, the combination of eNK cells and PD-L1 blockade partly increased ICAM1 expression on SCLC cells, compared to treatment with eNK cells alone (Supplementary Fig. 1 K– M). Notably, this combination therapy led to a slight decrease in HLA-ABC, HLA-E, and MICA/B in SBC5 (Supplemental Fig. 2A–G). These findings indicate that the combination of eNK cells and PD-L1 blockade resulted in a slight decrease in inhibitory and activating NK cell ligands in SCLC cells.

Effect of eNK cells plus PD-L1 blockade on the expression of $CD44^+CD90^+$ (stem cell-like markers) and $CD44^+CD56^+$ on SCLC

In this study, SCLC cells exhibited low expression of stem cell-like markers ($CD44^+CD90^+$). However, upon treatment with eNK cells alone, SCLC cells underwent a rapid phenotypic shift, characterized by an upregulation of stem cell-like markers ($CD44^+CD90^+$). Interestingly, the combination of PD-L1 blockade and eNK cell treatment led to a reduction in the expression of these stem cell-like markers ($CD44^+CD90^+$) on SCLC cell lines (Figs. 3A andB; Supplementary Fig. 2H). Moreover, this combination treatment notably reduced the co-expression of $CD44^+PD\text{-}L1^+$ on SCLC cell lines (Supplementary Figs. 3A–C). This finding indicates that the combination of PD-L1 checkpoint blockade plus eNK cell potential targets both stem-like characteristics and immunoevasive phenotypes in SCLC.

SCLC cell lines can express both neuroendocrine (NE; CD56) and non-neuroendocrine cell phenotypes (ML; CD44), and the coexisting NE^+ML^+ phenotype relates to the end stage and resistant phase of SCLC cells with a more SCLC stem-like phenotype [26]. In this study, DMS273 and H69 cell lines exhibit high co-express NE^+ML^+, while H82, SHP77, and H209 are NE^+ML^-. Next, SCLC cell lines were treated with eNK or eNK plus PD-L1 blockade. Flow cytometry measurement of NE and ML markers was used to characterize how treatments shifted the phenotypic identity of SCLC. This data demonstrated that the treatment with eNK plus PD-L1 blockade strongly suppressed the proportion of coexisting NE^+ML^+ SCLC during eNK treatment (Fig. 3C and D).

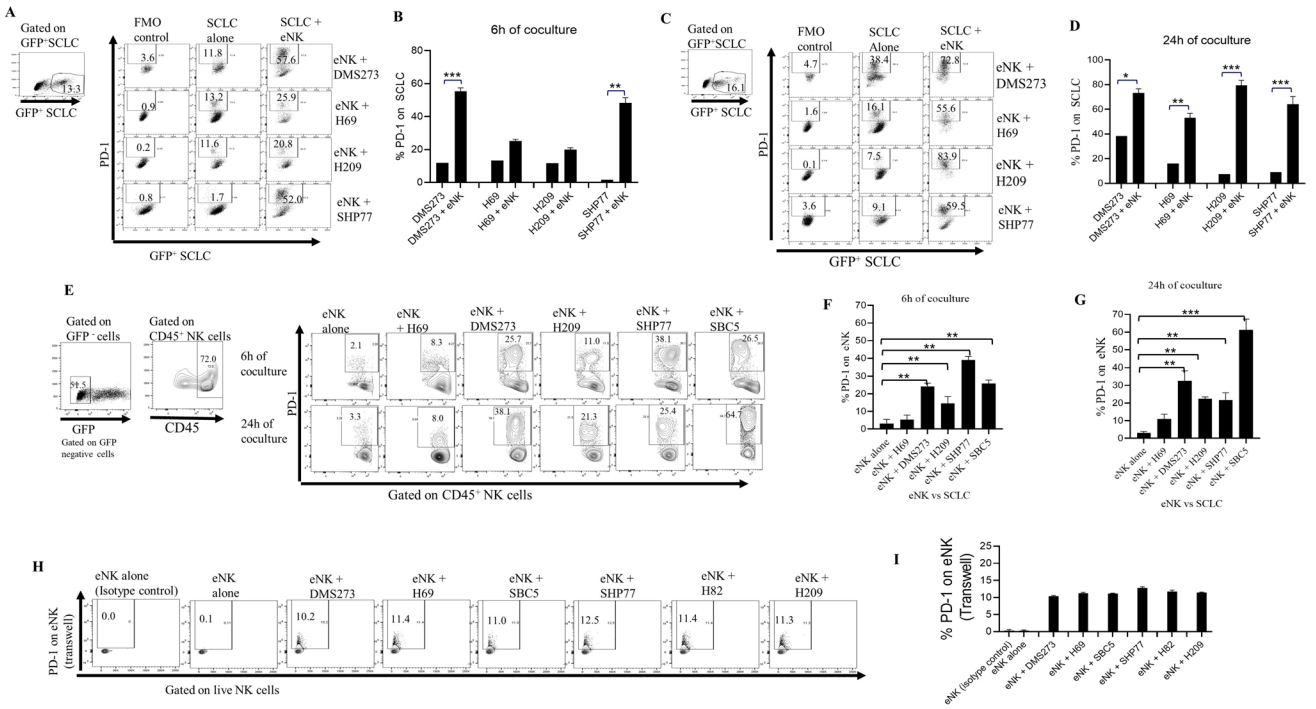

Fig. 2 PD-1 is upregulated on the surface of SCLC and eNK cells after co-culture. GFP-positive SCLC cell lines, including SBC-5, SHP77, DMS273, H69, and H209, were co-cultured with eNK cells to evaluate the percentages of PD-1 expression on both SCLC cell lines and eNK cells. (**A** and **B**) PD-1 expression on SCLC cell lines was analyzed after 6-h and (**C** and **D**) overnight co-culture with eNK cells using flow cytometry. Similarly, PD-1 expression on eNK cells was assessed following co-culture with SCLC cell lines at (**E** and **F**) 6-h and (**E** and **G**) overnight time points, also measured by flow cytometry. Differences between groups were analyzed using two-way ANOVA. $^{*}P<0.05$, $^{**}P<0.012$, and $^{***}P<0.001$. (**H** and **I**) PD-1 expression on eNK cells co-cultured with SCLC cell lines in transwell systems was analyzed. Data are representative from three independent experiments (n = 3)

PD-L1 blockade robustly increased the cytotoxic effect of eNK cells against SCLC cell lines

To explore the effect of PD-L1 blockade on the eNK-mediated killing of SCLC cells, we next evaluated the cytotoxic effect by LDH-release assay, CFSE-based assay, CD107a degranulation assays, and IFN-γ intracellular staining. The combination of PD-L1 blockade plus eNK resulted in a significant increase in the cytotoxic activity of eNK cells, as evidenced by increased levels of LDH release (Fig. 4A, and supplementary Fig. 3D), and greater cytotoxicity against CFSE-labelled SCLC cell lines (Fig. 4B and C). Additionally, the combination of PD-L1 blockade plus eNK cells was associated with increased CD107a (Fig. 4D and E) and IFN-γ (Figs. 4F and G) expression in eNK cells against SCLC cell lines, with statistical significance observed in IFN-γ expression in the DMS273 cell line. However, no significant changes were detected in the expression of granzyme B (Supplementary Fig. 3E and F) or perforin (Supplementary Fig. 3G and H). These results indicate that PD-L1 blockade synergistically enhances the killing effect on SCLC cells in combination with eNK cells.

Combining eNK with PD-L1 checkpoint blockade exhibits potent efficacy in vivo

Next, using DMS273, we established a human SCLC mouse model that accurately mimics human SCLC (Fig. 5A). All PBS control tumor-bearing mice showed rapid tumor growth, which led to death within 3 weeks. In contrast, tumor-bearing mice receiving eNK cells plus PD-L1 blockade exhibited significantly inhibited tumor growth and prolonged survival by nearly twice as long as mice in the PBS control group (Fig. 5B–D, and Supplementary Fig. 4A and B; ****, $P<0.0001$). Only mice receiving eNK cells plus PD-L1 blockade survived until 35 days after SCLC cell infusion (Fig. 5D; ****, $P<0.0001$). In addition, mice treated with eNK cells plus PD-L1 blockade had significantly lower levels of progastrin-releasing peptide (proGRP) than the mice in the other groups (Fig. 5E; **, $P<0.012$). ProGRP, a precursor of the neuropeptide gastrin-releasing peptide (GRP), is commonly secreted by SCLC cells [27, 28]. Circulating proGRP serves as a highly sensitive and specific biomarker in patients with SCLC [28–30], and is the most sensitive marker for discriminating SCLC from benign diseases of the lung [30]. These results suggest that

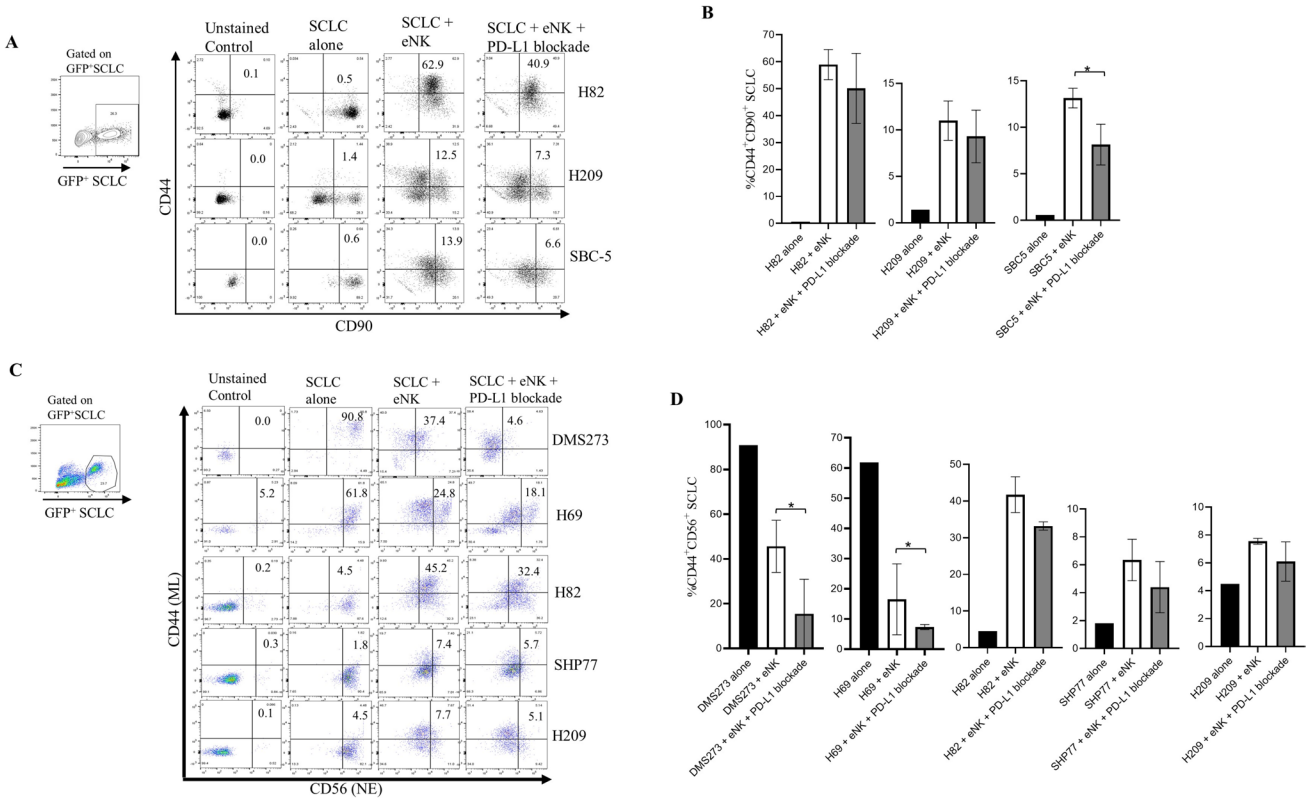

Fig. 3 eNK cells plus PD-L1 blockade greatly suppressed the stem cell population, as well as the neuroendocrine (NE) and non-neuroendocrine cell phenotypes (ML) coexisting in SCLC. (**A** and **B**) Quantification of stem cell-like markers (CD44 and CD90) expressed on small cell lung cancer (SCLC) cells, as assessed by flow cytometry. (**C** and **D**) Analysis of the co-expression of CD56 (NE) and CD44 (ML) markers in SCLC cell lines, evaluated using flow cytometry. Differences between groups were analyzed using two-way ANOVA. (*$P < 0.05$). Data are representative from three independent experiments (n = 3)

eNK cells plus PD-L1 blockade induces long-term systemic anticancer effects in a mouse model of SCLC. To analyze the potential functions, persistence, and homing of eNK cells in vivo, flow cytometry was performed on the liver, lungs, and tumors of mice. Infused NK cells were detected in the liver, lung, and tumor at 1, 3, and 22 days after infusion (Figs. 6A−D, and Supplementary Figs. 5, 6, 7, 8, 9, 10, 11, 12 and 13). However, the percentages of infused NK cells were lower in tumors (< 5%; Fig. 6A and D) compared to those in the liver (20% to 40%; Fig. 6A and B) or the lung (5% to 30%; Fig. 6A and C). Importantly, on day 22 after the final eNK cell infusion, mice were sacrificed, and single-cell suspensions from the tumor were prepared to assess the effector function of tumor-infiltrating eNK cells. Analysis revealed that eNK cell-infiltrating tumors in mice receiving the combined treatment of eNK cells plus PD-L1 blockade exhibited a markedly enhanced activation phenotype, with high expression of memory receptors such as NKG2C, activation receptors such as NKG2D, migration receptors such as CXCR4, and costimulatory molecules such as DNAM-1 compared to mice treated with eNK alone (Fig. 6E–G).

Additionally, in liver and lung tissues, infused NK cells in mice treated with eNK plus PD-L1 blockade showed an enhanced activation phenotype, characterized by high expression of DNAM-1, NKG2C, CXCR4, NKG2D, and CD16, compared to infused NK cells from mice treated with eNK cells alone (Fig. 7A− J, and Supplementary Figs. 5A and B, 7A and B, 10A and B, and 12A and B). These cells also exhibited upregulation of apoptosis ligands such as FasL, cytotoxicity markers such as IFN-γ and Granzyme-B, and proliferation markers such as Ki67, indicating highly cytotoxic phenotypes (Fig. 7K−R, and Supplementary Figs. 6A and B, 8A and B, 11A and B, and 13A and B). Notably, we noted a reduction in TIM3 checkpoint molecules in infused NK cells in the liver and lung of mice treated with eNK cells plus PD-L1 blockade compared to the infused NK cells in mice treated with eNK cells alone (Supplementary Fig. 9A and B). These data suggest that the infusion of PD-L1 blockade enhances the function and persistence of eNK cells even under in vivo conditions, allowing them to remain unaffected by factors in the TME for an extended period, which is strongly correlated with their

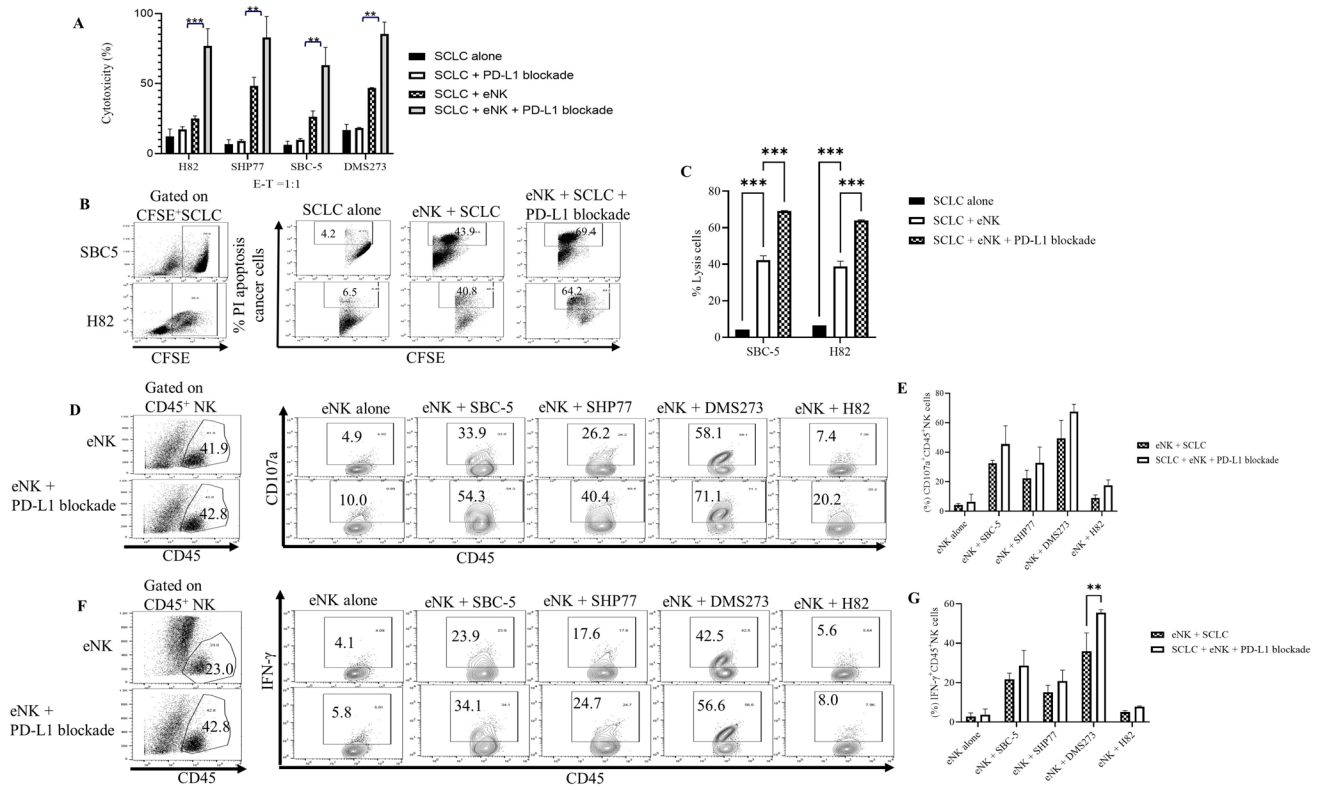

Fig. 4 PD-L1 blockade robustly increases the cytotoxicity of eNK cells against SCLC cell lines. (**A**) The killing capacity of eNK cells against SCLC was investigated using an LDH-release cytotoxicity assay. (**B**) FACS plots and (**C**) Bar graphs illustrating the cytotoxic activity of eNK cells against CFSE-labelled SCLC cell lines. (**D**) FACS analysis and (**E**) Bar graph quantification of CD107a expression in eNK following coculture with target SCLC cell lines. (**F**) FACS analysis and (**G**) Bar graph showing the percentage of IFN-γ.$^{+}$ eNK following coculture with target SCLC cell lines. Differences between groups were analyzed using two-way ANOVA. Data are representative from three independent experiments (n = 3)

improved anticancer effects by suppressing the production of immunosuppressive factors in the microenvironment.

Discussion

In this study, we demonstrated that the combination of NK cells and anti-PD-L1 mAbs has a synergistic effect against SCLC. Following co-culture of SCLC cell lines with NK cells, we observed increased expression of PD-L1 in both SCLC cell lines and NK cells. Additionally, following co-culture, NK cells and SCLC cell lines showed high levels of PD-1 expression. Moreover, the combination of eNK cells plus atezolizumab demonstrated increased cytotoxicity against target SCLC cells, a higher proportion of IFN-γ, increased CD107a degranulation marker expression, as well as higher levels of SCLC stem cell ($CD44^{+}CD90^{+}$) suppression and non-neuroendocrine/mesenchymal-like SCLC phenotype suppression.

Previous studies have examined how cancer cells that overexpress PD-L1 molecules respond to the activities of NK cells and other immune cells, enabling them to survive and grow [7, 15]. Our results are consistent with those of previous studies, in that the SCLC cell lines showed overexpression of PD-L1 molecules after co-culturing with eNK cells. Additionally, cancer cells secrete numerous immunosuppressive factors, such as TGF-β, VEGF, and IL-10, to form a strong TME during the development process or to evade immune cells. These inhibitory factors induce the expression of PD-1 on T cells, NK cells, and other immune cells, leading to the suppression of the immune response against cancer and facilitating its evasion from immune attack [7, 31]. Consistently, in this study, co-culturing eNK cells with SCLC resulted in high PD-1 expression on both eNK cells and SCLC cell lines. It has been reported that PD1 can be expressed at high levels on tumor-infiltrating NK cells, and PD1-mediated inhibition of NK cells occurs through the engagement of PD1 on NK cells by PD-L1 on tumor cells, resulting in reduced NK cell responses that lead to the generation of more aggressive tumors in vivo [32, 33]. Our data strongly confirmed that the expression of PD-1 on NK cells is caused by the contact between NK cells and SCLC cells, and we believe that, in the in vivo TME, tumor cells can induce highly expressed PD-1 on NK cells.

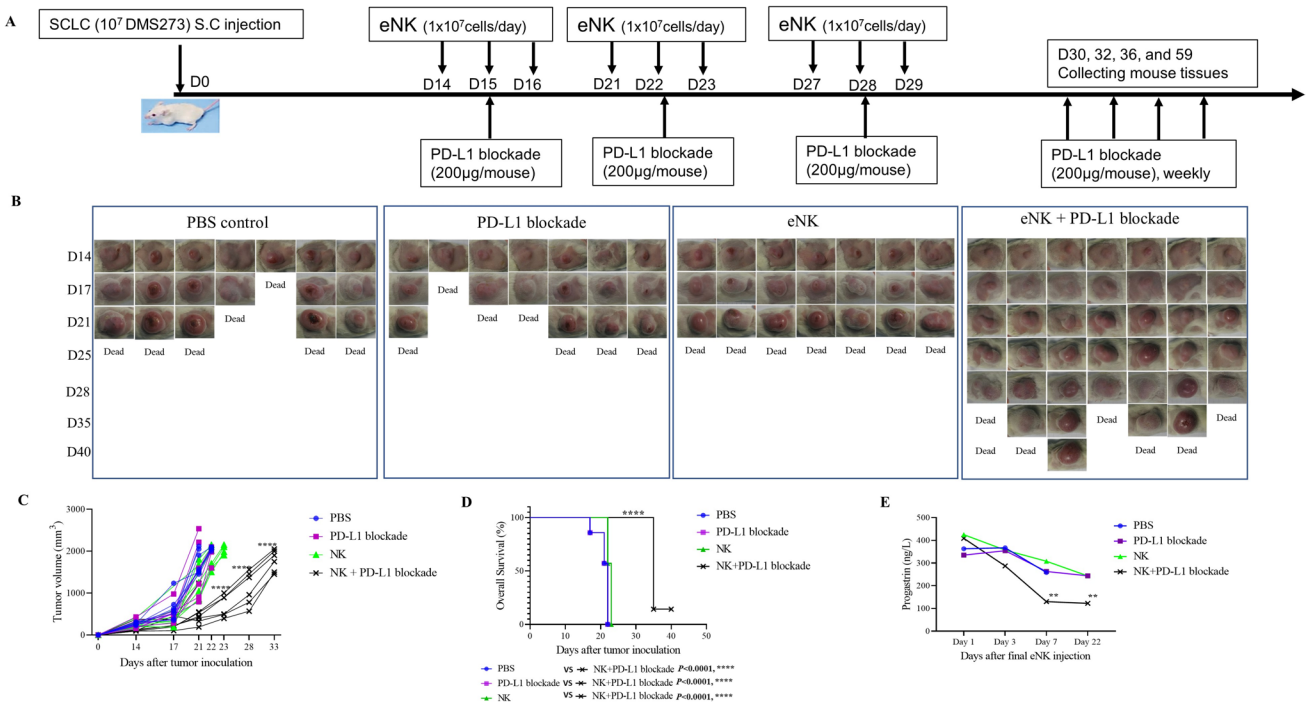

Fig. 5 eNK cells plus PD-L1 blockade exerts potent anticancer effects in the SCLC xenograft model. (A) Schematic summarizing the treatment of SCLC-bearing mice with eNK cells combined with PD-L1 blockade. (B) Representative images of injected SCLC tumors from mice (7 mice per group) treated with eNK cells plus PD-L1 blockade. (C) Growth rates of the tumor mass (****$P < 0.0001$), and (D) survival time of tumor-bearing mice (**$P < 0.01$). (E) Serum levels of pro-gastrin-releasing peptide (ProGRP) in the serum of vaccinated mice (**$P < 0.012$, ****$P < 0.0001$). Differences between groups were analyzed using two-way ANOVA. Data are representative from two independent experiments

Importantly, we confirmed the role of PD-L1 checkpoint blockade in mediating NK cell cytotoxicity against SCLC, which expands our understanding of how anti-PD-L1 antibodies synergistically enhance NK cell cytotoxicity against diverse SCLCs expressing PD-L1. Our findings suggest that PD-L1 checkpoint blockade can enhance the cytotoxicity of NK cells against SCLC. In addition, this study clarified the phenomenon of how tumor cells in TME express both PD-1 and PD-L1 and suggested that the optimal timing for anti-PD-L1 mAb treatment is a critical point to increase the efficient activation of NK cells against tumor cells.

Trogocytosis, which is responsible for protein transfer, exerts a substantial effect on several cellular processes [34, 35]. In cancer, trogocytosis is associated with reduced immune responses and failure of immunotherapy [34, 35]. Indeed, cancer cells can transfer or acquire inhibitory receptors, such as PD-L1 and CTLA-4, to or from immune cells via the trogocytosis pathway, which can then inhibit immune cell activities and decrease the efficacy of immunotherapy [34, 36]. Here, we speculated that trogocytosis may be a mechanism by which PD-L1 becomes localized on the surface of NK cells (Figs. 1H and IJ). SCLC cells overexpressed PD-L1 molecules after co-culture with NK cells, resulting in the transfer of PD-L1 to NK cells, indicating that PD-L1 was trogocytosed to NK cells via SCLC cells. Interestingly, a previous study [18] identified a novel and unique subset of NK cells characterized by the surface expression of PD-L1 in a fraction of patients with acute myeloid leukemia. Binding of anti-PD-L1 mAb to PD-L1$^+$ NK cells induced strong antitumor activity in vitro and in vivo. Consistently, our results demonstrated that in the presence of PD-L1 checkpoint blockade, NK cell-induced cytotoxicity was significantly increased against SCLC cell lines.

SCLC is characterized by aggressiveness, heterogeneity, high rates of metastasis, and drug resistance, suggesting its enrichment in cancer stem cells (CSCs) [3, 37]. In addition, previous reports [26, 38] have demonstrated that spontaneously occurring neuroendocrine (NE) and non-neuroendocrine cell phenotypes coexist and cooperate to promote metastasis and drug resistance in SCLC. NK cells have long been known for their ability to reject allogeneic hematopoietic stem cells, and there is growing evidence that NK cells can target and eliminate quiescent or non-proliferating cells such as CSCs [39]. Our results demonstrated that eNK cells plus PD-L1 checkpoint blockade significantly suppressed the stem cell population (CD44$^+$CD90$^+$) as well as the coexisting NE and non-neuroendocrine cell phenotypes coexisting (CD44$^+$CD56$^+$) in SCLC.

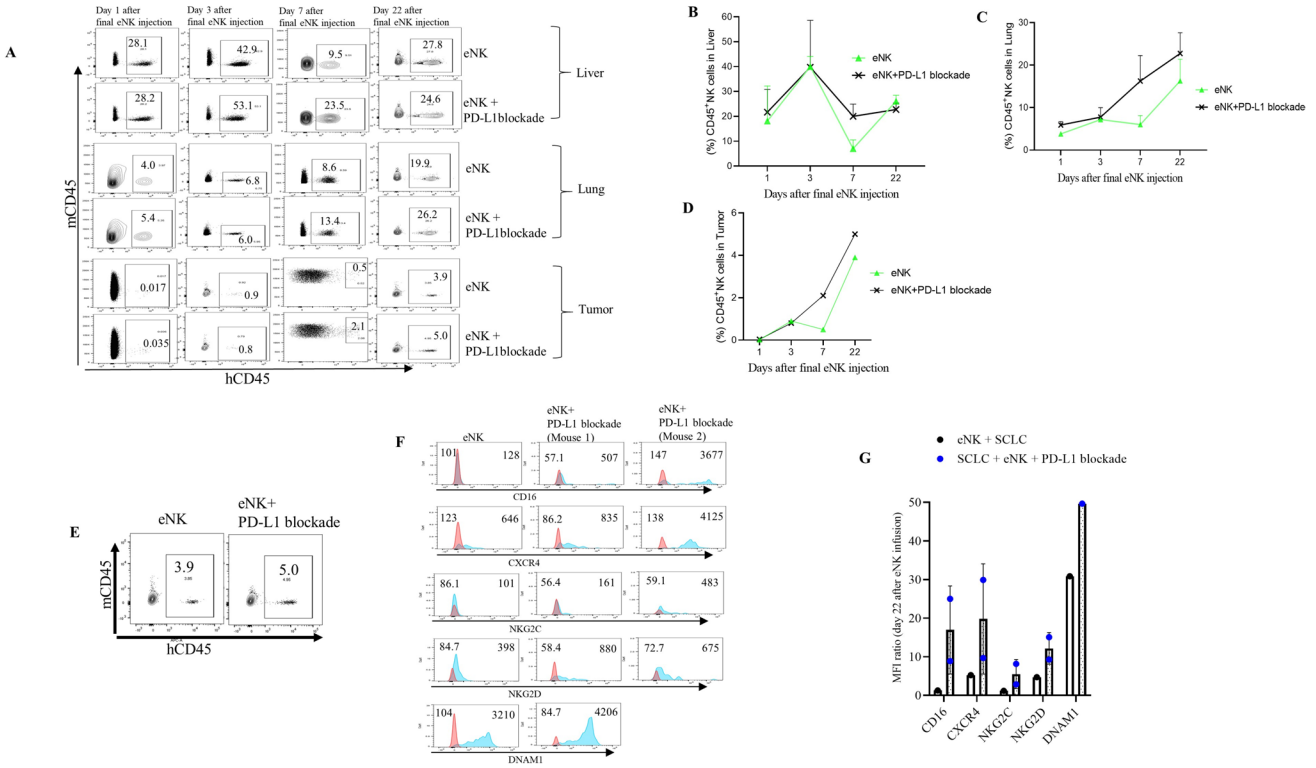

Fig. 6 PD-L1 blockade improves the in vivo effector function and persistence of eNK cells in SCLC-bearing mice. (**A**) Dot plots showing the in vivo distribution of $CD45^+$ eNK cells in the liver, lung, and tumor of vaccinated mice. Bar graphs quantifying the distribution of circulating eNK cells in the liver (**B**), lung (**C**), and tumor (**D**), assessed via flow cytometry. On day 22 after final eNK cell infusion, mice were sacrificed, and single cell suspensions from the tumor were used to assess the effector function of tumor infiltrating eNK cells. (**E**) Representative FACS dot plot showing the in vivo distribution of $hCD45^+$ eNK cells in the tumor of vaccinated mice. (**F**) FACs histograms provide the expression of activation markers on eNK cells in the tumor of vaccinated mice, including memory receptors such as NKG2C, activation receptors such as CD16 and NKG2D, migration receptors such as CXCR4, and costimulatory molecules such as DNAM-1. (**G**) The MFI ratios (MFI of samples/MFI of isotype controls) of each sample are shown as bar graphs. Data are derived from two representative mice (n = 2)

Furthermore, in our in vivo NSG mouse model, we demonstrated that the combination of eNK and PD-L1 checkpoint blockade strongly inhibited tumor growth, reduced serum ProGRP levels, and prolonged the survival of vaccinated mice.

This suggests that the antitumor effects are mediated directly by NK cells following PD-L1 signaling within the TME. Importantly, the infusion of eNK cells alone or in combination with PD-L1 checkpoint blockade did not cause graft-versus-host disease or cytokine release syndrome in our in vivo model. We also investigated the anti-tumor effects of the combination therapy at three time points (days 1, 3, 7, and 30 after final eNK cell injection) to assess the kinetics of eNK cell activation and distribution in vivo following injection. Compared to eNK injection alone, the combination of eNK cells plus PD-L1 checkpoint blockade resulted in a significant increase in activating receptors (NKG2D, DNAM1, and CD16), the memory-like phenotype marker NKG2C, the migration marker CXCR4, and cytotoxicity markers such as IFN-γ, FasL, GRZB, and Ki67. We demonstrated the role of PD-L1 checkpoint blockade in mediating the antitumor effect of eNK cells in this setting, revealing a new strategy for establishing an increased and prolonged immune response by NK cells in the TME of SCLC. This approach is likely applicable to some, but not all, tumors, and it explains how immune therapy with anti-PD-L1 mAb can be effective in tumors with low PD-L1 expression.

Atezolizumab greatly induces $CD8^+$ T cell activation, which is a key mechanism underlying its efficacy in treating certain types of cancer with high PD-L1 expression [40, 41]. Our findings clearly demonstrate that a combination of eNK cells and atezolizumab has a synergistic effect against SCLC, providing new hope to patients with SCLC with commonly low-expressing PD-L1 molecules. In the clinic, we expect that the eNK cell treatment induces PD-L1 molecules highly expressed on SCLC cells, and the addition of atezolizumab blocks the interaction of PD1/PD-L1, which would not only synergistically activate eNK cells but also local patients's $CD8^+$ T cells to efficiently kill the SCLC.

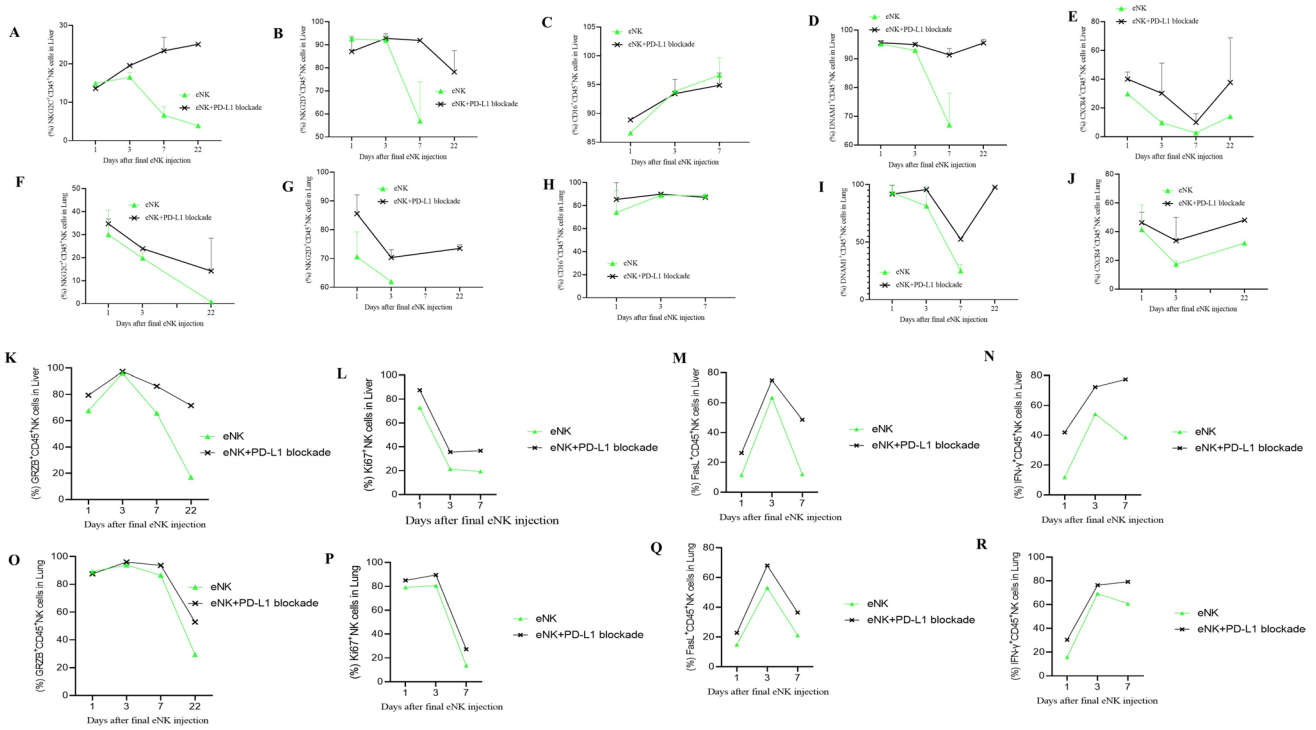

Fig. 7 PD-L1 blockade improves in vivo cytotoxicity function of eNK cells in SCLC-bearing mice. Bar graphs showing the phenotypic characteristics of circulating eNK in the lung and liver, including memory receptors such as NKG2C (**A** and **F**), activation receptors such as NKG2D (**B** and **G**) and CD16 (**C** and **H**), costimulatory molecules such as DNAM-1 (**D** and **I**), and migration receptors such as CXCR4 (**E** and **J**). Data are derived from two representative mice (n = 2). Bar graphs quantifying the levels of cytotoxicity markers in of circulating eNK cells in the liver and lung of vaccinated mice, such as granzyme-B (**K** and **O**), proliferation markers such as Ki67 (**L** and **P**), apoptosis ligands such as FasL (**M** and **G**), and IFN-γ (**N** and **R**). Data are derived from single representative mice at each time point (n = 1)

In conclusion, our results demonstrated that binding of anti–PD-L1 mAb to PD-L1^{+}SCLC and PD-L1^{+} NK cells induced strong antitumor activity in vitro and in vivo. We also reveal that PD-L1 checkpoint blockade therapy has a unique therapeutic function in treating cancers with low PD-L1 expression, such as SCLC, though its action on NK cells. This novel mechanism of directly activating NK cells with anti-PD-L1 mAb therapy may explain the efficacy of PD-L1 checkpoint inhibitors in some PD-L1-negative tumors. These findings provide a framework for developing more advanced immunotherapeutic modalities for future clinical trials and extending the survival of patients with advanced SCLC.

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Author contributions MCV, JJL, and IJO designed the study; MCV and VTN performed the experiments and interpreted the data; HJO, SHJ and WKB contributed intellectually to the study; MCV and IJO wrote the manuscript; and JJL and IJO supervised the study.

Data availability The data generated in this study are available within the article and supplementary data files.

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

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