Propofol improves the function of natural killer cells from the peripheral blood of patients with esophageal squamous cell carcinoma

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Abstract. Postoperative immunosuppression is associated with the recurrence and metastasis of esophageal squamous cell carcinoma (ESCC). Propofol is a commonly used intravenous anesthetic and has been reported to be associated with immunosuppression; however, little is known about its effect on innate immune cells during the postoperative period in patients with ESCC. The aim of the present study was to investigate the effects of propofol on the phenotype and cytotoxicity of natural killer (NK) cells derived from the peripheral blood of patients with ESCC. The percentage, phenotype and function of NK cells were compared between patients with ESCC and healthy volunteers using flow cytometry. NK cells were negatively sorted using magnetic beads and cocultured with propofol to assess changes in phenotype and function. The results revealed that the percentage of NK cells was significantly increased in the peripheral blood of patients with ESCC, while their activity and cytotoxicity were impaired. NK cells were successfully separated from peripheral blood *in vitro* and it was demonstrated that propofol enhanced their activity by influencing the expression of activating or inhibitory receptors. Furthermore, propofol was able to increase the cytotoxicity of NK cells from the peripheral blood of patients with ESCC. These results suggest that propofol is able to improve the function of NK cells in patients with ESCC and may therefore be an appropriate anesthetic for ESCC surgery.

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Introduction

Esophageal carcinoma is one of the most common digestive tract-derived malignancies worldwide (1,2). Esophageal carcinoma is comprised of squamous carcinoma and adenocarcinoma (3). A high incidence of esophageal squamous carcinoma (ESCC) has been reported in China (4); in 2015, 429,200 cases of cancer were reported in China, with 281,400 mortalities (5). Of these cases, 259,000 were ESCC, making it the fifth most prevalent malignant tumor (6). Tumor recurrence and metastasis are leading causes of mortality in patients with ESCC (7). It has previously been reported that immunosuppression is associated with tumor recurrence and metastasis (8,9), however the underlying mechanism remains to be elucidated.

It is well established that immunosuppression is associated with the recurrence and metastasis of multiple tumors (10). The immune response of infiltrating lymphocytes in tumor tissues is impaired by certain components in the tumor microenvironment, which leads to tumor recurrence and metastasis (11,12). Furthermore, it has been reported that restoration of the immune system may improve the survival of patients with ESCC (13). It has been demonstrated that various anesthetic agents exert different but important effects on immune cells in patients with tumors (14). It may therefore be beneficial to select an anesthetic that improves immunosuppression in patients with tumors.

Innate immunity is the body's first line of defense against infection, tumors and virus invasion (15). Compromised innate immunity has been reported in a number of tumor types and is associated with the survival of patients with tumors, suggesting a crucial role of innate immunity in the inhibition of tumors (16). Natural killer (NK) cells are an important component of the innate immune system, they are typically cluster of differentiation (CD)3^{-}CD56^{+}cells, which and are primarily distributed in the peripheral blood, with 10-15% in lymphocytes (17,18). NK cells are responsible for immune surveillance without antigen presentation and major histocompatibility complex (MHC) restriction (19). Once activated, NK cells are able to recognize target cells rapidly and release cytotoxic effector molecules to trigger the immune response (20). The aim of the present study was to evaluate the effects of

propofol on NK cells derived from the peripheral blood of Patients with ESCC. Propofol is one of the most commonly used anesthetics and has been reported to exert analgesic and anti-inflammatory effects (21).

Materials and methods

Patients and tissue sample. A total of 5 ml fresh peripheral blood was collected from 15 patients with ESCC (age, 39-43 years; 10 male, 5 female) prior to surgery and 15 healthy volunteers (age, 26-35 years; 11 male, 4 female) at the Affiliated Hospital of Southwest Medical University (Luzhou, China) between January and May 2017. All patients were diagnosed with primary ESCC and had not undergone therapy. The exclusion criteria for patients with ESCC were as follows: Previous chemotherapy or radiotherapy, chronic disease, infectious disease or multi-primary cancer. Exclusion criteria for the healthy patients included chronic, autoimmune or genetic diseases. Patient clinical data is presented in Table I. The present study was approved by the Ethics Committee of the Southwest Medical University and informed consent was obtained from each patient.

Cell isolation. Fresh peripheral blood of patients with ESCC was stored in anticoagulant tubes (BD Biosciences, Franklin Lakes, NJ, USA) and used to isolate mononuclear lymphocytes and NK cells as previously described (22). In brief, mononuclear lymphocytes were isolated from 5 ml peripheral blood using Ficoll separation medium (Tianjin Yanyang Biochemical Co., Ltd., Tianjin, China). The cells were centrifuged at 2,000 x g for 20 min at room temperature and the middle layer was mononuclear lymphocytes used for the NK separation. NK cells were negatively sorted using a human NK cell separation medium kit purchased from BD Biosciences according to the manufacturer's protocol. NK cells were negatively sorted using the MagniSort™ Human NK cell Enrichment kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Isolated NK cell suspensions were then identified using flow cytometry analysis characterized by CD3-CD56+.

Flow cytometry analysis. NK cells were incubated with the appropriate surface antibodies for 15 min at room temperature. Cells were fixed with 4% formaldehyde at room temperature for 10 min and subsequently permeabilized for 40 min using Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's protocol. Permeabilized cells were stained with antibodies against Ki67, granzyme B and perforin for 15 min at room temperature. For intracellular staining of interferon (IFN)γ and tumor necrosis factor (TNF)α, NK cells were stimulated for 4 h at 37˚C in 5% CO_2 with 50 ng/ml phorbol myristate acetate and 1 mg/ml ionomycin in the presence of Golgistop (BD Biosciences) prior to staining with fluorescence labeling antibodies (Table II) for 15 min at room temperature. A flow cytometer was used for analysis with FAC Suite version 1.0.3.2942 (BD Biosciences).

In vitro coculture of NK cells with propofol. NK cells were separated from the peripheral blood and cultured at 37˚C in RPMI 1640 medium with 10% fetal bovine serum (both

BD Biosciences) in the presence of 100 U/ml interleukin (IL)2, 100 U/ml IL12 and 100 U/ml IL15 for 48 h. Cells were subsequently incubated in RPMI 1,640 medium with 50 μmol/l propofol (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 24 h at 37˚C. The purity of NK cells was >90% as confirmed by flow cytometry. The markers of NK cells (NKG2D, NKp30, NKp44, NKG2A, CD3, CD56, CD16, CD226, CD158b, NKp46, Ki67, CD107a, IFNγ, granzyme B, perforin and TNFα) were detected using a flow cytometer.

NK cells cytotoxicity assay. Following coculture with propofol, the number of NK cells was calculated. NK cells were subsequently mixed with K562 and Eca109 cells (Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China), each at a ratio of 1:5. Apoptosis was evaluated using flow cytometry. NK cells were treated using the fluorescein isothiocyanate Annexin V Apoptosis Detection kit I (BD Biosciences) according to the manufacturer's protocol. Cells positive for Annexin V alone were in early apoptosis, cells positive for propidium iodide (PI) alone were necrotic and cells positive for Annexin V and PI were in late apoptosis. Untreated NK cells from patients with ESCC were used as the control for this experiment.

Statistical analysis. Data are presented as the mean ± standard deviation. Student's t-test was used to compare between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

The phenotype and function of NK cells from the peripheral blood of patients with ESCC. NK cells have been reported to be important for suppressing the recurrence and metastasis of tumor cells. Furthermore, it has been reported that immunosuppression occurs in the tumor microenvironment as well as the peripheral blood (23). The results of flow cytometry revealed that the percentage of NK cells gated by CD3-CD56+ in the peripheral blood of patients with ESCC (21.6±0.89%) was significantly increased compared with the control (11.8±0.54%; Fig. 1). Furthermore, the percentage of NKT cells gated by CD3+CD56+, another type of innate immune cell (24), was significantly increased in the peripheral blood of patients with ESCC compared with the control group (3.99±0.43 vs. 1.15±0.10; Fig. 1), indicating that the innate immune response activity was increased.

NK cell activation is known to be controlled by activating and inhibitory receptors (25). In the present study, the expression of activating and inhibitory receptors was assessed to evaluate the activity and function of NK cells. The results revealed that the expression of activating receptors of CD16, NKG2D, CD226 and p30 were significantly increased in compared with the control group (Fig. 2). However, no significant difference in p44, p46, CD158b, G2A, granzyme B, perforin, IFNγ and TNFα expression was observed (Fig. 3). The cytotoxicity of NK cells to K562 was assessed by detecting the expression of CD107a in NK cells. It was demonstrated that the number of CD107a+ NK cells was significantly increased in patients with ESCC compared with the healthy controls, suggesting that the cytotoxicity of NK

Table I. Clinical data of the patients with esophageal squamous cell carcinoma.

Index	Number of patients
Sex	
Male	3
Female	12
Age	
≤60	5
≥60	10
Tumor size	
≤5 cm	9
≥5 cm	6
Location	
Middle	11
Lower	4
Differentiation	
Well	8
Moderate	7
Poor	0
Invasion depth	
Outer layer	2
Inner layer	13
Lymph node metastases	
With	7
Without	8

cells is reduced in patients with ESCC (Fig. 2). Ki67+ cells were also counted to evaluate the proliferation potential of NK cells. No significant difference in proliferation was observed between groups (Fig. 3). These data suggest that the function of NK cells in the peripheral blood of patients with ESCC is impaired compared with those from healthy subjects.

Purification and identification of NK cells. To further investigate the potential influence of propofol on NK cells in ESCC, NK cells were negatively isolated using magnetic beads sorting (26). CD3-CD56+ cells associated with the lymphocyte gate by forward scatter (FSC) and side scatter (SSC) on a dot-plot were defined as NK cells. The results demonstrated that the number of sorted NK cells from the peripheral blood of patients with ESCC or healthy volunteers was $3.5\pm0.25\times10^5$ or $2.7\pm0.11\times10^5$, respectively, while the purity of NK cells was >90% and the percentage of CD3+ T cells was <1%, suggesting that the sorting of NK cells was successful and suitable for subsequent experiments (Fig. 4).

Surface markers of NK cells as detected by flow cytometry. To investigate the impact of propofol on NK cells, the phenotype of isolated NK cells cocultured with propofol was assessed. A concentration of 50 μmol/l propofol was selected as this has been reported to be an appropriate concentration for studying its function (27). The expression of activating and inhibitory receptors on the surface of NK cells was determined using

flow cytometry. The results revealed that the percentage of several activating receptors was significantly increased in NK cells cocultured with propofol compared with the control group, including CD16, NKp30, NKp44 and NKG2D (Fig. 5). Conversely, the expression of inhibitory receptors CD158b and NKG2A was significantly decreased in NK cells treated with propofol compared with the control group (Fig. 5). No significant differences in NKp44, NKp46 and CD226 expression was observed (data not shown). These data suggest that propofol may enhance the activity of NK cells in ESCC by regulating the equilibrium between activating and inhibitory receptors.

Propofol enhances the expression of granzyme B and IFN-γ in NK cells. It is well established that activated NK cells destroy tumor cells mainly via cytotoxicity effector molecules, including granzyme B, perforin, IFN-γ and TNF-α (28). The results of flow cytometry revealed that the expression of granzyme B and IFN-γ was significantly increased following incubation with propofol (Fig. 6), suggesting that NK cell cytotoxicity was increased. These data suggest that propofol may promote the cytotoxicity of NK cells from patients with ESCC.

Propofol promotes the proliferation potential of isolated NK cells. The influence of propofol on the proliferation potential of isolated NK cells was assessed by detecting expression of Ki67 through flow cytometry. The results demonstrated that the number of Ki67+ NK cells was significantly higher following propofol stimulation compared with control cells (Fig. 7). These data suggest that propofol may promote the proliferative potential of NK cells in patients with ESCC.

Propofol enhances the function of NK cells in vitro. Based on previous findings, the cytotoxicity of NK cells cocultured with propofol to tumor cells was assessed *in vitro* using apoptosis analysis. K562 was selected as the target cell line as it does not express MHCI molecules (29). The apoptosis rate of K562 cells cocultured with NK cells stimulated by propofol was significantly higher compared with the control group (Fig. 8). To further investigate the cytotoxicity of NK cells to ESCC cells, the apoptosis rate of Eca109 cells incubated with NK cells was assessed. Consistent with K562, NK cells cultured with propofol exerted a greater cytotoxic effect on Eca109 cells compared with the control (Fig. 8). These data suggest that propofol may enhance the cytotoxicity of NK cells from the peripheral blood of patients with ESCC.

Discussion

Elucidating the effect of anesthesia on immune inhibition during the postoperative period is essential for preventing tumor metastasis and improving the prognosis of patients with ESCC (30). Although anesthetic agents have been demonstrated to affect tumor recurrence and metastasis (31), the impact of anesthetics on anti-tumor immune cells is not well understood. In the present study, NK cells were successfully isolated from the peripheral blood of patients with ESCC and it was confirmed that propofol is able to increase the activity of NK cells by regulating the expression of receptors and

Table II. Antibodies used for flow cytometry.

Antibody	Company	Cat. no.	Conjugate	Dilution
NKG2D	BD Biosciences	552364	Percp-cy5.5	1:40
NKp30	BD Biosciences	558407	PE	1:40
NKp44	BD Biosciences	558563	PE	1:40
NKG2A	R&D Systems, Inc.	FAB1059C	Percp	1:40
CD3	BD Biosciences	555916	Fluorescein isothiocyanate	1:40
CD56	BD Biosciences	555518	Allophycocyanin	1:40
CD16	BD Biosciences	557744	PE-cy7	1:40
CD226	BD Biosciences	559789	PE	1:40
CD158b	BD Biosciences	559785	PE	1:40
NKp46	BD Biosciences	557991	PE	1:40
Ki67	BD Biosciences	561283	PE-cy7	1:40
CD107a	BD Biosciences	555801	PE	1:40
Interferon-γ	BD Biosciences	559326	PE	1:40
Granzyme B	BD Biosciences	561142	PE	1:40
Perforin	BD Biosciences	563762	Percp-cy5.5	1:40
Tumor necrosis factor α	BD Biosciences	560679	Percp-cy5.5	1:40

BD Biosciences is located in Franklin Lakes, NJ, USA. R&D Systems, Inc. is located in Minneapolis, MN, USA. NK, natural killer; PE, phycoerythrin; Percp, peridinin-chlorophyll-protein; CD, cluster of differentiation.
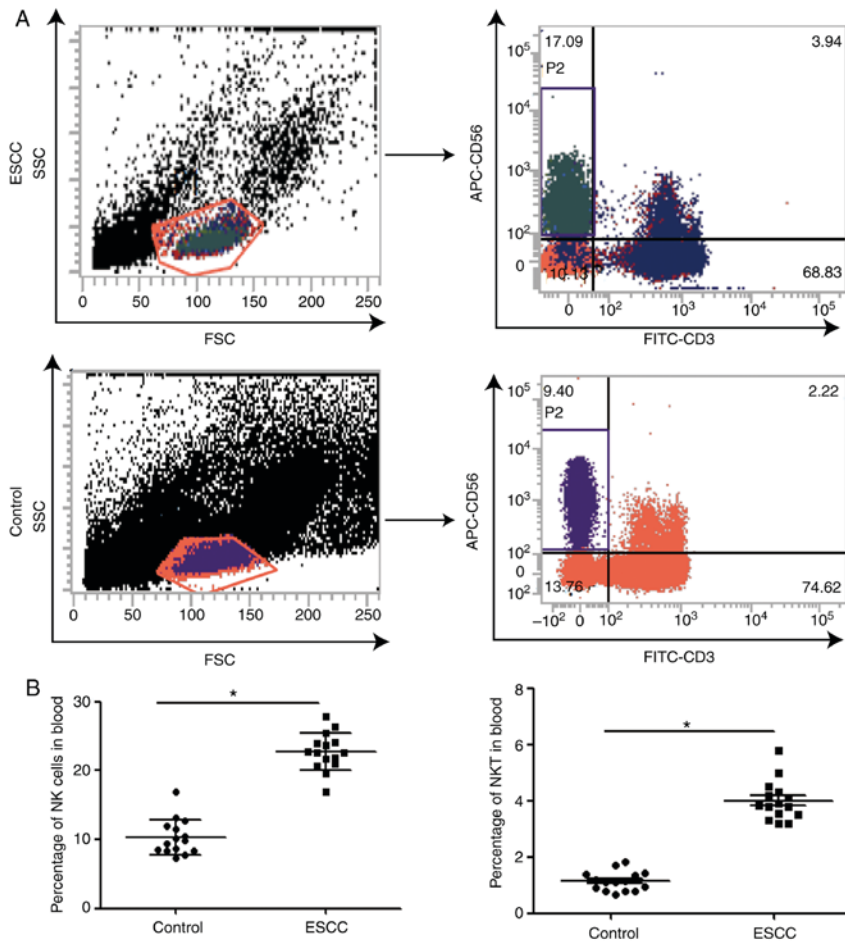

Figure 1. Percentages of NK, NKT and T cells in the peripheral blood of patients with ESCC and control subjects, respectively. (A) Representative flow cytometry plots of NK, NKT and T cells, gated by CD3-CD56+, CD3+CD56+ or CD3+, respectively. (B) Percentages of NK and NKT cells in patients with ESCC and control subjects. *P<0.05. NK, natural killer; NKT, natural killer T; ESCC, esophageal squamous cell carcinoma; CD, cluster of differentiation; SSC, side-scattered light; FSC, forward scatter; APC, allophycocyanin; FITC, fluorescein isothiocyanate.
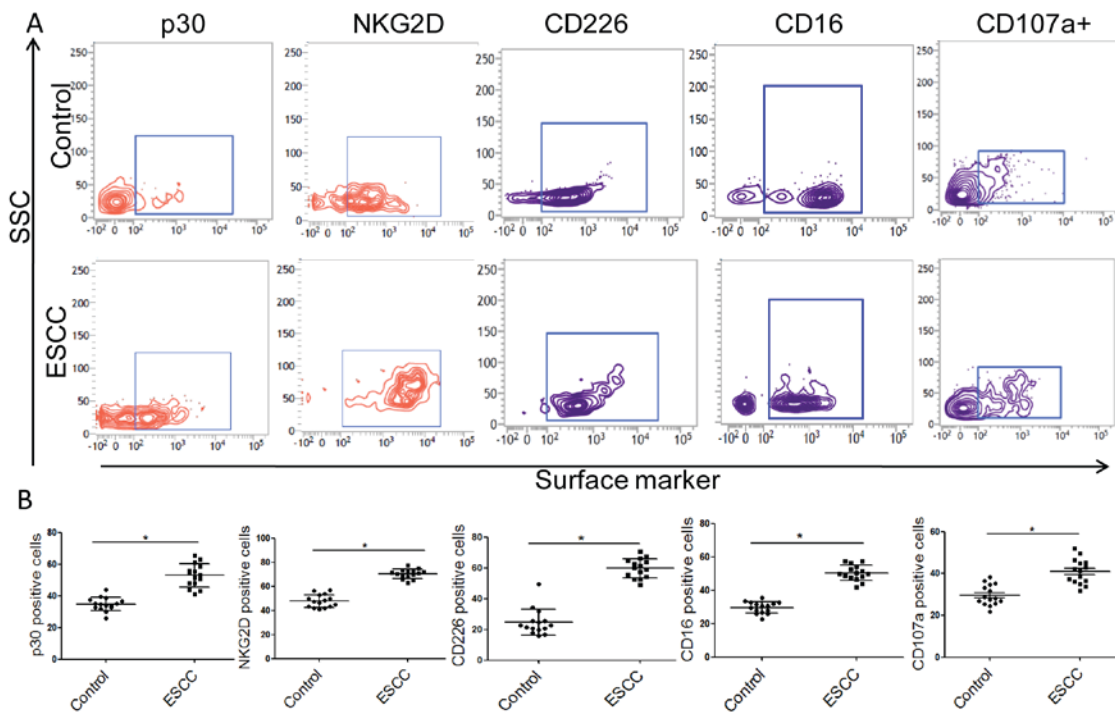
Figure 2. Phenotype analysis of NK cells from the peripheral blood of patients with ESCC and control subjects. (A) Representative flow cytometry images and (B) quantitative analysis of the activating and inhibitory receptors of NK cells. *P<0.05. p30, NKG2D, CD226, CD16 and CD107a. NK, natural killer; ESCC, esophageal squamous cell carcinoma; NKG2D, NK group 2, member D; CD, cluster of differentiation; SSC, side-scattered light.
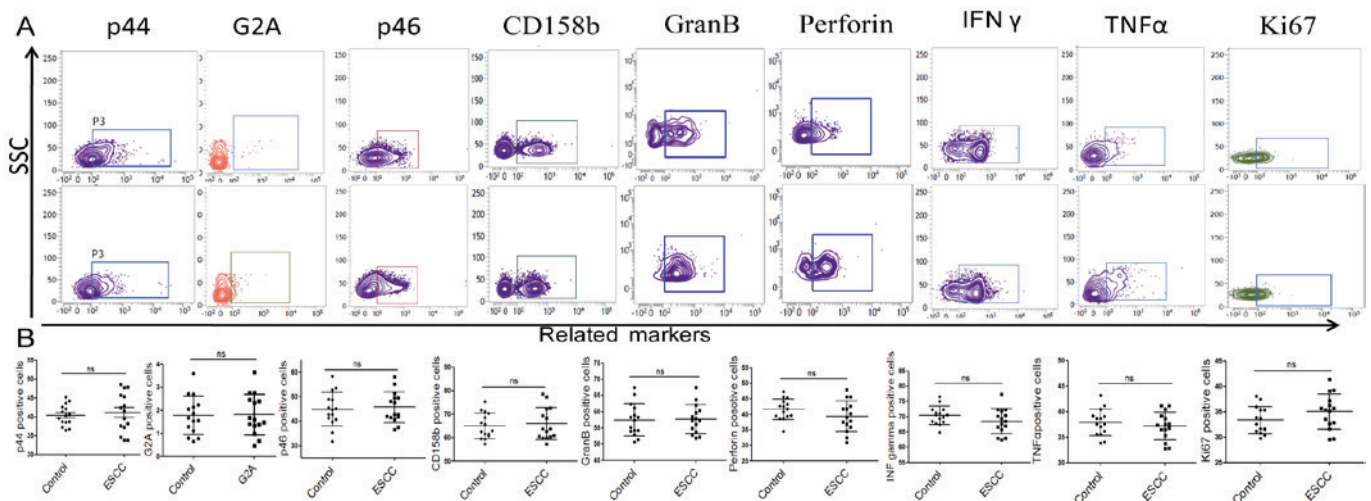

Figure 3. The nonsense phenotype of NK cells from the peripheral blood of patients with ESCC and control subjects. (A) Representative flow cytometry images and (B) quantitative analysis of activating and inhibitory receptors and effect molecules in NK cells. (B) The column showed that the expression of p44, G2A, p46, CD158b, granzyme B, perforin, TNFα, IFNγ and Ki67. NK, natural killer; ESCC, esophageal squamous cell carcinoma; G2A, G-protein coupled receptor 132; CD, cluster of differentiation; TNF, tumor necrosis factor; IFN, interferon; SSC, side-scattered light; ns, no significance.

cytotoxicity effect molecules. Furthermore, propofol enhances the cytotoxicity of NK cells to ESCC cells *in vitro*, indicating that it serves as a promotion effector for the postoperative immune system in ESCC.

NK cells are a group of innate lymphoid cells, which serve a crucial role in the inhibition of multiple tumors (32). A novel tumor therapy technique, adoptive immunotherapy, based on NK cells has been investigated in human trials (33). The activation or inhibition of NK cells has been reported to depend on the recognition of altered tumor cells by the activation or inhibition of receptors on the NK cell surface. Therefore, screening factors

that influence the expression of NK cell surface receptors is a critical area of immunotherapy for tumors (34). Anesthetics have been reported to be associated with innate immune system regulation, providing a novel perspective that anesthesia was implicated with tumor recurrence and metastasis by regulation of immune response (35). The cytotoxicity of NK cells from patients with breast cancer receiving propofol-remifentanil anesthesia with postoperative ketorolac analgesia was clearly lower compared with those receiving sevoflurane-remifentanil anesthesia with postoperative fentanyl analgesia (36); Inada *et al* (37) reported that propofol promotes the expression
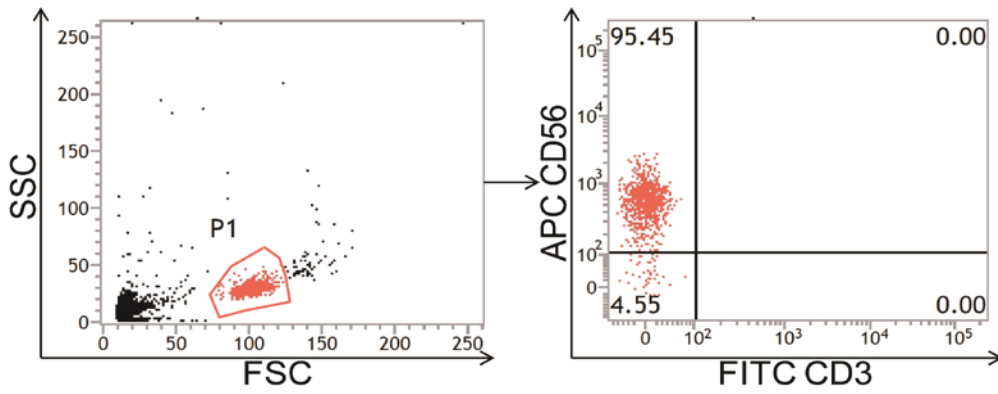
Figure 4. Representative flow cytometry images demonstrating the purity of isolated NK cells. NK, natural killer; CD, cluster of differentiation; SSC, side-scattered light; FSC, forward scatter; APC, allophycocyanin; FITC, fluorescein isothiocyanate.
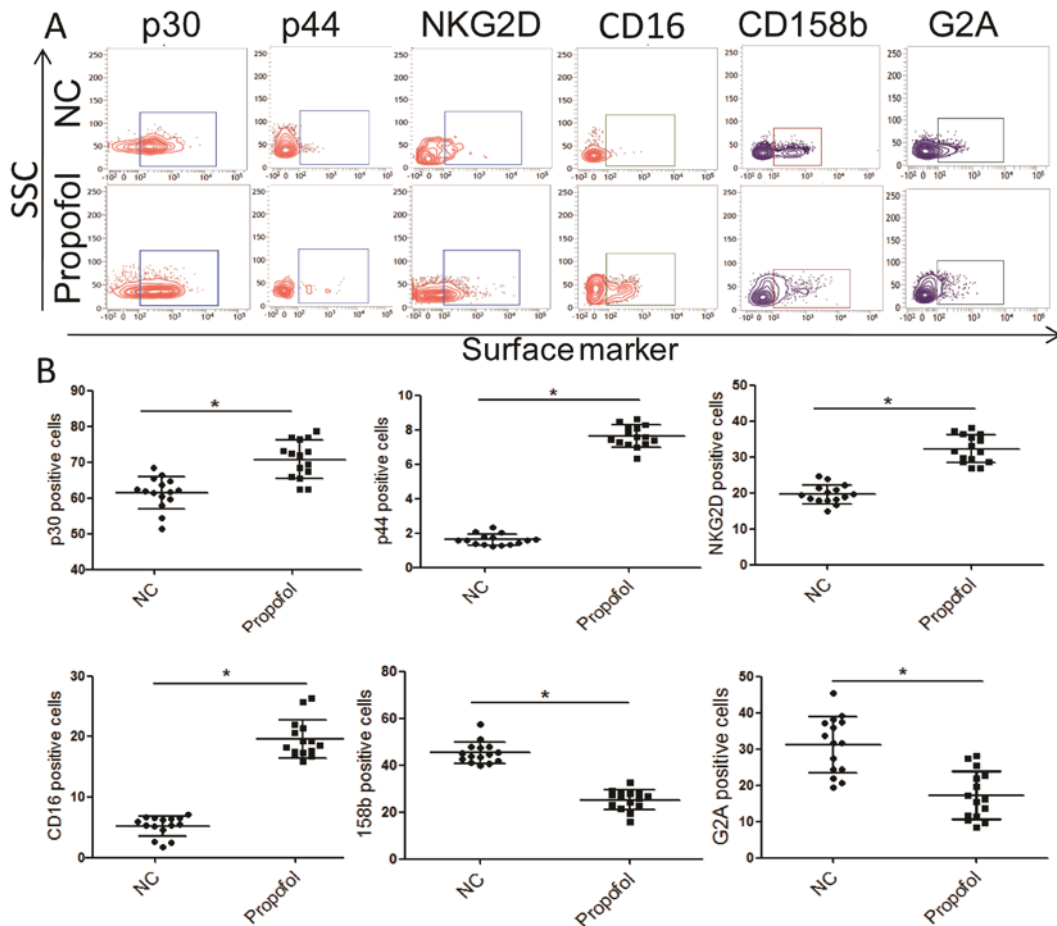

Figure 5. Effect of propofol on the expression of activating and inhibitory receptors of NK cells *in vitro*. (A) Representative flow cytometry images and (B) quantitative analysis of p30, p44, NKG2D, CD16, CD158b and G2A expression. *P<0.05. NK, natural killer; NKG2D, NK group 2, member D; CD, cluster of differentiation; G2A, G-protein coupled receptor 132; NC, negative control; SSC, side-scattered light.

of IFNγ in NK cells by suppressing prostaglandin E2 (37). This suggests that propofol is associated with the regulation of NK cytotoxicity; however, its impact on the expression of activation and inhibitory receptors remains unclear. Consistent with previous studies (38), the percentage of NK cells from patients with ESCC was increased compared with the control, which may be a response to tumorigenesis. The phenotype and cytotoxicity of NK cells was investigated and the results demonstrated that NK cells from patients with ESCC had a higher expression of

activating receptors (p30, NKG2D, CD226 and CD16) compared with the control, suggesting that NK cells from the peripheral blood of patients with ESCC were activated. Conversely, it has previously been reported that NK cells patients with tumors had impaired function (39,40). These contradictory results may be because some crucial signaling pathway downstream of activating receptors also serves a role in the regulation of NK cells,.

To further evaluate the effect of propofol on NK cells, isolated NK cells from patients with ESCC were incubated
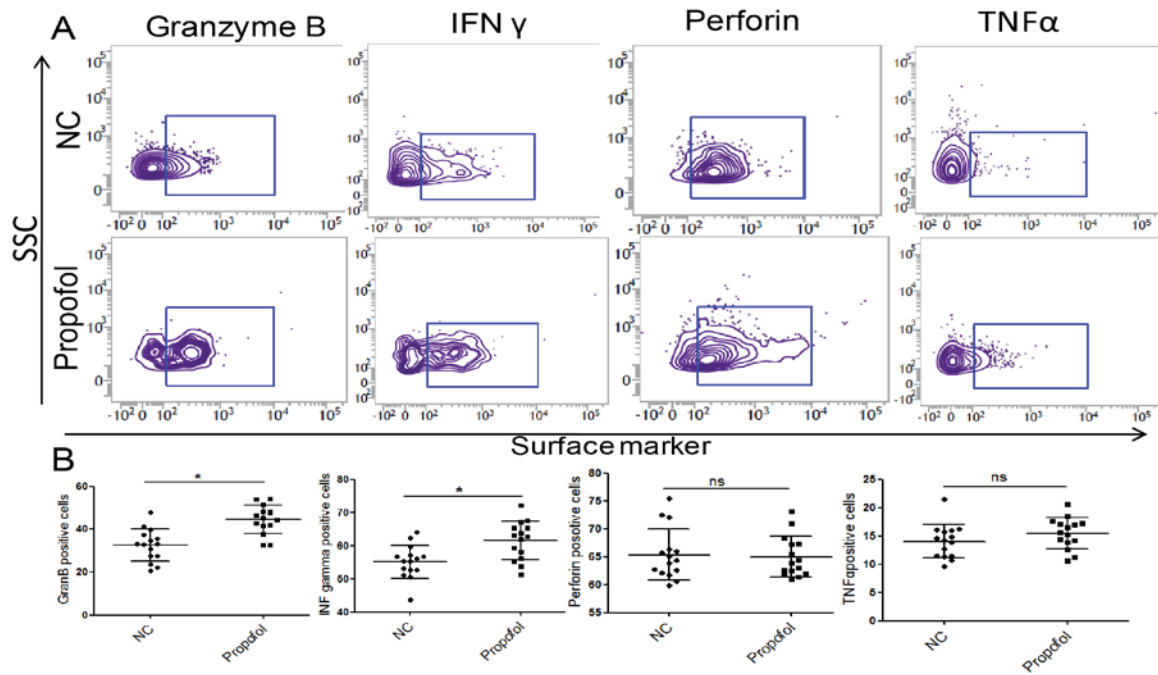
Figure 6. Effect of propofol on the functional characteristics of NK cells from patients with ESCC. (A) Representative flow cytometry images and (B) quantitative analysis of granzyme B, IFNγ, perforin and TNFα in NK cells from patients with ESCC cocultured with propofol. *P<0.05. NK, natural killer; ESCC, esophageal squamous cell carcinoma; IFN, interferon; TNF, tumor necrosis factor; SSC, side-scattered light; NC, negative control; ns, no significance.
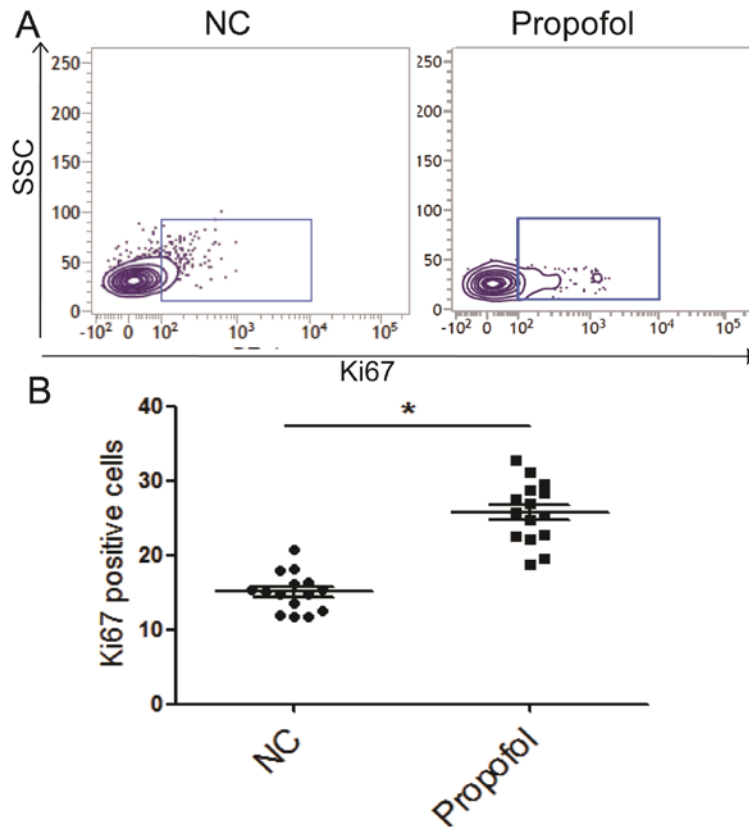

Figure 7. Propofol promotes the proliferation of NK cells from patients with ESCC. (A) Representative flow cytometry images and (B) quantitative analysis of Ki67 expression in NK cells from patients with ESCC cocultured with propofol. *P<0.05. NK, natural killer; ESCC, esophageal squamous cell carcinoma; SSC, side-scattered light; NC, negative control.

with propofol followed by analysis via flow cytometry. The results revealed that propofol increased the expression of activating receptors (p30, NKG2D, p44, CD16) expression and suppressed inhibitory receptors (CD158b, NKG2A). The cytotoxicity of NK cells from patients with ESCC was also enhanced, as indicated by the increased expression of IFNγ
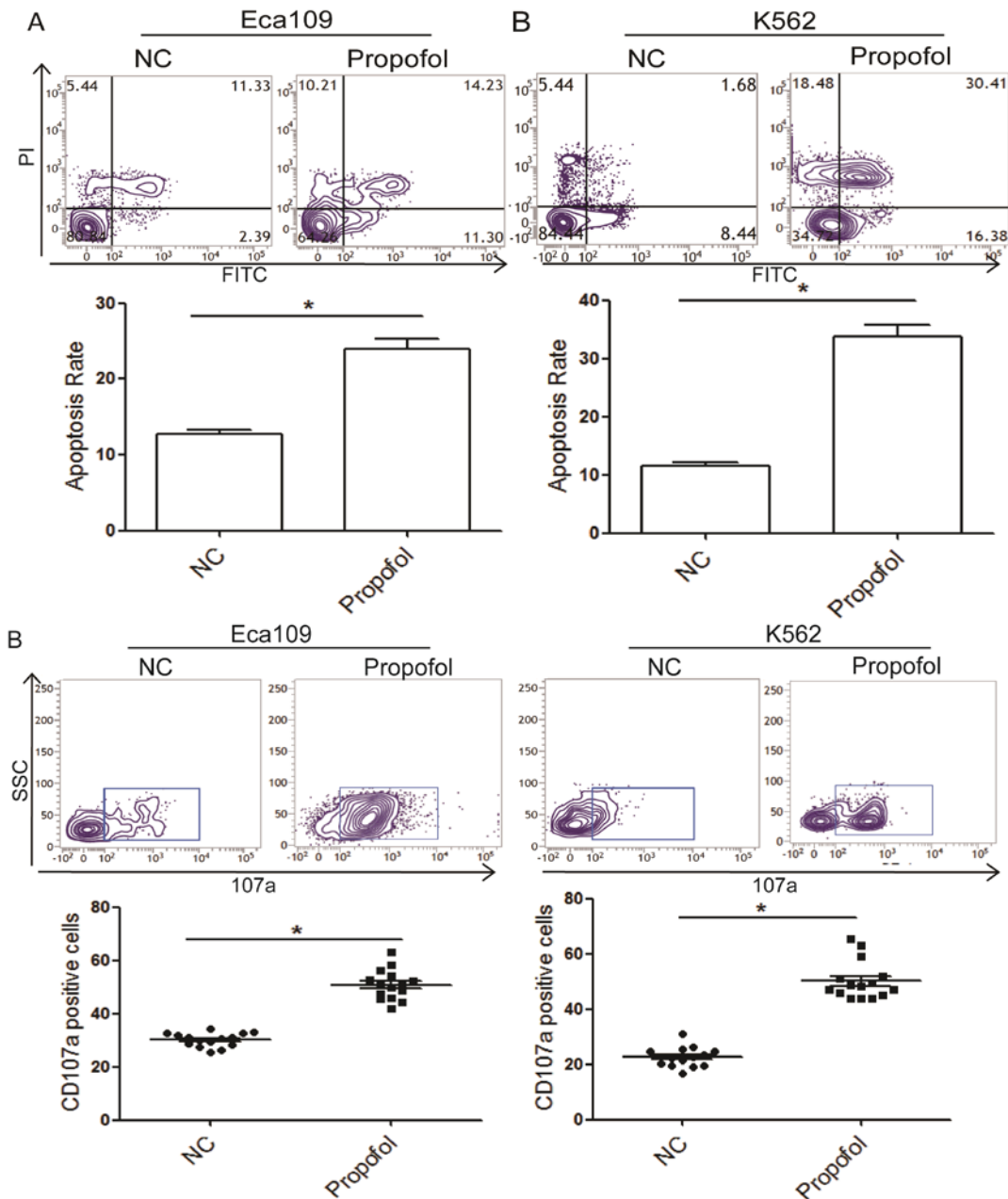
Figure 8. Propofol enhances the cytotoxicity of NK cells to K562 and Eca109 cells, respectively. (A) Representative flow cytometry images and quantitative analysis of the apoptosis rate of K562 and Eca109 cells treated with propofol. (B) Representative flow cytometry image for CD107a positive rate analysis for K562 and Eca109 cells cocultured with propofol. *P<0.05. NK, natural killer; ESCC, esophageal squamous cell carcinoma; CD, cluster of differentiation; PI, propidium iodide; NC, negative control; FITC, fluorescein isothiocyanate; SSC, side-scattered light.

and granzyme B. Ki67 was also upregulated in NK cells stimulated with propofol, indicating that propofol improves the proliferation potential of NK cells. Although data within the present study indicated that propofol probably promoted the activation of NK cells from patients with ESCC, the cytotoxicity of NK cells still needs to be confirmed. Blocking the activating interaction between activating receptors and matched ligands led to impaired NK cell function in the presence of high expression of activating receptors. The cytotoxic effects of NK cells on K562 and Eca109 were investigated and it was revealed that propofol-stimulated NK cells increased apoptosis in K562 and Eca109 cells. Furthermore, a significant increase in CD107a + NK cells, which are characteristic of degranulation, was observed

following propofol stimulation. These data suggest that propofol is able to enhance the cytotoxic effects of NK cells from the peripheral blood of patients with ESCC *in vitro*. In the present study, the number of patients recruited was insufficient to analyze the association between NK cell markers and clinicopathological characteristics, including lymph node metastasis and TNM stage. Furthermore, the function of propofol on NK cells should be verified using an *in vivo* mouse model in the future.

In conclusion, the results of the present study demonstrated that propofol is able to enhance the function of NK cells from the peripheral blood of patients with ESCC. Based on these results, propofol may have the potential to improve postoperative immunosuppression in patients with ESCC.

Acknowledgements

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XW designed the study and wrote the paper. MZ and JCD performed the experiments. YZ, JW, TX and DZ collected the samples and performed flow cytometry. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants in the present study. The study was approved by the Ethics Review Board at Southwest Medical University (Luzhou, China).

Consent for publication

All patients provided written informed consent for the publication of their data.

Competing interests

The authors declare that there were no competing interests.

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