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ORIGINAL INVESTIGATION

The T cell activating properties and antitumour activity of Staphylococcal Enterotoxin-like Q

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Abstract

Staphylococcal enterotoxins (SEs), as typical superantigens, exhibit promising antitumour activity in the clinic, but their unavoidable side effects related to fever and emesis seriously limit their application for the treatment of malignant tumours. Fortunately, the identification of Staphylococcal enterotoxin-like toxins (SEls), which possess amino acid sequences similar to those of classical SEs but exhibit no or low emetic activity, has provided a set of potential immunomodulatory candidates for cancer therapy. The aim of this study was to examine the effect of SElQ on lymphocyte activation and to further demonstrate its antitumour activity both in vitro and in vivo. High-purity SElQ was successfully harvested, and in vitro results confirmed that SElQ can significantly activate mouse- and human-derived lymphocytes in a dose-dependent manner, particularly $CD4^+$ and $CD8^+$ T cells, which showed significant increases in both percentage and absolute number. Further examination revealed that in addition to the originally recognized TCR *V*β5 and 21, TCR *V*β14, 17 and 18 were activated in SElQ-induced human PBMCs. Moreover, the expression of IL-2 and IFN-γ was significantly upregulated in vitro and in vivo after SElQ treatment. Based on the findings that SElQ induces lymphocyte activation and cytokine release, we then confirmed its antitumour activity both in vitro and in vivo. The data showed that treatment with a low concentration of SElQ (30 μg/mouse) could inhibit the growth of tumours by approximately 30% and no significant toxicity was observed. Taken together, our results demonstrated that SElQ can significantly induce T cell activation and cytokine release and further elicit substantial antitumour activity and thus provide support for the potential application of SElQ in cancer immunotherapy.

Keywords Immunotherapy · Lymphocyte · Staphylococcal enterotoxin · Staphylococcal enterotoxin-like Q · Superantigen

Introduction

Staphylococcal enterotoxins (SEs) are representative of a group of evolutionarily related molecules known as superantigens (SAgs) because of their outstanding immunostimulatory capability [1, 2]. Unlike conventional antigens, SEs can bind outside the peptide groove of major histocompatibility complex class II (MHC II) molecules instead of being processed by antigen-presenting cells (APC) and can then vigorously stimulate the proliferation of T cells bearing specific T cell receptor (TCR) beta-chain variable (*V*β) regions [3, 4]. As a result, SEs are capable of stimulating a large proportion (up to 5–20%) of T cells (particularly $CD4^+$ and $CD8^+$ T cells) and initiating a massive release of cytokines, including IL-1, IL-2, IFN-γ, TNF-α, and TNF-β, which consequently induce strong activation of the immune system in vivo [4–6].

During the past decade, classical SEs (SEA, SEB and SEC2) and their mutants have been widely studied as

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effective therapeutic agents for antitumour immunotherapy [7, 8]. However, accompanying their SAg activity, classical SEs can induce vomiting, diarrhoea and fever, which severely limits their clinical application [9, 10]. Fortunately, based on recommendations of the International Nomenclature Committee, further research has defined staphylococcal enterotoxin-like toxins (SEls), a subgroup of the SE family that possesses similar amino acid sequences to classical SEs but exhibits no or low emetic activity. To date, a series of SEl toxins, including SElJ, SElK-Q, SElU, SElV and SElX have been identified [11].

In this study, we have successfully constructed pET28a-SElQ and obtained a high-purity SElQ protein using Ni^{+}-affinity magnetic beads (SElQ is a His-tagged recombinant protein). Further results further showed that SElQ can significantly stimulate the proliferation of mouse-derived splenic lymphocytes and human-derived peripheral blood mononuclear cells (PBMCs), and the specific TCR Vβ subgroups activated by SElQ were examined by qRT-PCR. The significant antitumour activity observed both in vitro and in vivo suggested that SElQ could be used as a potential candidate agent for tumour treatment in the clinic.

Materials and methods

Strains, plasmid and animals

SElQ-producing *Staphylococcus aureus* S6 strain, *Escherichia coli* strain BL21 (DE3), *Escherichia coli* strain DH(5α), the expression vector pET-28a (Fig. S1A) and tumour cells were provided by our laboratory; SPF female BALB/c mice (aged 6–8 weeks, weighting 22–26 g) were purchased from Vital River Laboratory Animal (Beijing, China) (License No SCXK (Jing) 2012–0001).

The peripheral blood samples used in this study were harvested with the informed consent of the donors, and the experimental procedures performed with the peripheral blood samples were approved by the Ethics Committee of Xinxiang Medical University. Furthermore, the mice handling and experimental procedures were performed with approval from the Animal Care Committee of Xinxiang Medical University in accordance with the guidelines established by the Chinese Council on Animal Care.

Production of high-purity SElQ

The detailed procedures used for SElQ production are described in supplementary methods (Fig. S1 and S2) and mainly include two steps: (1) construction and verification of the prokaryotic expression vector pET28a-SElQ and (2) expression and purification of SElQ. All SElQ proteins used in this study were His-tagged.

Preparation of mouse splenic lymphocytes and human PBMCs

For the isolation of mouse splenic lymphocytes, the spleens from naive mice were removed aseptically, and splenocytes were obtained by squeezing the organs in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA). The resulting cell suspension was filtered through a 100-μm stainless steel mesh, and the erythrocytes were osmotically lysed by a brief incubation in a solution containing 155 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA-Na2. After two washes, the splenic lymphocytes were resuspended and adjusted in complete medium (RPMI 1640 with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin) to a density of 5×10^6 cells/ml.

For the isolation of human PBMCs, heparinized venous blood donated by volunteers ($n = 5$) was immediately transported to the laboratory within 8 h. The PBMCs were diluted with the same volume of PBS and separated from the diluted peripheral blood by density gradient centrifugation, and the residual erythrocytes were lysed as described above. After two washes, the PBMCs were resuspended and adjusted in complete medium to a density of 1×10^6 cells/ml.

Proliferation assays

The adjusted mouse splenic lymphocytes (5×10^6 cells/ml) and human PBMCs (1×10^6 cells/ml) were seeded into the wells of 96-well plates (Corning, USA) at 200 μl/well, and co-cultured with serial tenfold dilutions of SElQ (final concentrations: 0.001, 0.01, 0.1, 1, and 10 μg/ml) at 37 °C with 5% CO_2 for 72 h. Complete medium served as a blank control, cells that were incubated with BSA (final concentration: 10 μg/ml) (Sigma, St. Louis, MO, USA) served as the negative control, and cells that were incubated with ConA (final concentration: 5 μg/ml) (Sigma, St. Louis, MO, USA) served as the positive control. The proliferative responses were determined through a conventional methylthiazol tetrazolium (MTT) (Sigma, St. Louis, MO, USA) assay, and the stimulation effect is reported as the stimulation effect index (SI): SI = (Abs value in experimental groups − background)/(Abs value in negative control groups − background).

Flow cytometry

The adjusted mouse splenic lymphocytes (5×10^6 cells/ml) were seeded into the wells of six-well plates, at 2 ml/well, and co-cultured with SElQ (final concentration: 1 μg/ml) at 37 °C in 5% CO_2 for 72 h. The cells were then collected,

counted using a cell counter (Countstar, China), washed twice in PBS, and resuspended in 100 µl of PBS. The cells were subsequently incubated with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibodies (eBioscience, San Diego, CA, USA) at 4 °C in the dark for 30 min; isotype PE and FITC-conjugated IgG (eBioscience, San Diego, CA, USA) were used as isotype controls. The samples were then washed with PBS and analysed using a Guava easyCyte cytometer.

ELISA

The adjusted mouse splenic lymphocytes (5×10^6 cells/ml) and human PBMCs (1×10^6 cells/ml) were incubated with serial tenfold dilutions of SElQ (final concentrations: 0.001, 0.01, 0.1, 1, and 10 µg/ml) at 37 °C in 5% CO_2 for 72 h. The culture supernatants were collected by centrifugation, and the production of IL-2 and IFN-γ was examined using the appropriate enzyme-linked immunosorbent assay (ELISA) kits (Xinbosheng, Beijing, China) according to the manufacturer's instructions.

BALB/c mice were injected i.v. from the tail vein with different concentration of SElQ (10, 30 and 50 µg/mouse, respectively, 4 mice per group), and mice injected with an equal volume of PBS served as the control. At different time points (24, 48, and 72 h), blood samples (100 µl) were collected into heparin-treated tubes from the tail vein of the mice, heated with an infrared lamp for 5 min and diluted with an equal volume of PBS. The diluted serum samples (100 µl) were then centrifuged and isolated, and the production of IL-2 and IFN-γ was examined using ELISA kits.

qRT-PCR

The adjusted mouse splenic lymphocytes (5×10^6 cells/ml) were seeded into the wells of six-well plates, at 2 ml/well, and stimulated with SElQ (final concentration: 1 µg/ml) at 37 °C in 5% CO_2 for 48 h. The total RNA from the stimulated splenic lymphocytes was extracted using the Ultrapure RNA Kit (ComWin, Beijing, China) according to the manufacturer's instructions, and cDNA was obtained from 1 µg of total RNA using the PrimeScript® RT Master Kit (Takara, Beijing, China). qRT-PCR was then performed using SYBR® Premix Ex Taq™ II Kit (Takara, Beijing, China) and ABI Prism 7500 system (Applied Biosystems, Norwalk, Connecticut, USA) according to the manufacturer's instructions. The primers specific for mouse β-actin, granzyme A and granzyme B [12, 13] that were used in this study are shown in Table 1. Data analysis was performed as described elsewhere using the comparative CT method ($2^{-\Delta\Delta Ct}$), and β-actin was used as the comparator. The results are presented as fold changes relative to the control, which was set to a value of one.

Human PBMCs (1×10^6 cells/ml) were cultured with SElQ (1 µg/ml) for 48 h, total RNA isolation and cDNA preparation were performed as mentioned above, and qRT-PCR was performed based on a previously described method [14]. The absolute copy number of each *V*β and *C*β region was extrapolated from the corresponding standard curve, and the percentage of each *V*β was calculated using the following equation:

$$\%V\beta_n = \left(V\beta_n/C\beta\right) \times 100.$$

In vitro antitumour activity assays

Human PBMCs were used as effector cells, and tumour cells BT474 (human breast cancer cell) and A431 (human epidermal cancer cell) were separately used as the target cells to test the antitumour activity of SElQ in vitro. Briefly, the target cells (BT474, 8×10^3 cells/100 µl or A431, 3×10^3 cells/100 µl) mixed with the effector cells (PBMCs, 2×10^5 cells/50 µl) were seeded in 96-well plates. PBS (50 µl) was added to the control wells, and serially diluted SElQ (50 µl, final concentrations: 0.1, 1 and 10 µg/ml) was separately added to the experimental SElQ-treated wells. The background wells contained RPMI 1640 medium only, and PBMCs stimulated with SElQ (final concentrations: 0.1, 1 and 10 µg/ml) were used as the blank group. After incubation for 48 h at 37 °C with 5% CO_2, the inhibition of tumour cells growth was determined using MTT assay as described above and the following formula: tumour cell growth inhibition (%) = {1 − [(Abs value of the SElQ-treated group − Abs value of the background) − (Abs value of the

Table 1 Primer sequences

Gene	Oligonucleotide sequence (5′-3′)		References
β-Actin	Sense	TGGAATCCTGTGGCATCCATGAAAC	[12]
	Antisense	TAAAACGCAGCTCAGTAACAGTCCG	
Granzyme A	Sense	ATCTGTGCTGGCGCTTTGA	[13]
	Antisense	ACTTAGATCTCTTTCCCACGTTACAGT	
Granzyme B	Sense	CGATCAAGGATCAGCAGCCT	[13]
	Antisense	CTTGCTGGGTCTTCTCCTGTTCT	

blank group − Abs value of the background)]/(Abs value of the control group − Abs value of the background)} × 100%.

Since the MTT assay can only examine the proliferation or metabolic activity of co-cultured cells (PBMCs and tumour cells), we further examined the effect of the cytokine cocktail derived from SElQ-treated PBMCs on the proliferation or cell death of A431 cells through non-contact co-culture. Briefly, A431 cells (5×10^4 cells/800 µl) were seeded in 24-well plates for 12 h, PBMCs (1×10^6 cells/200 µl) stimulated with serially diluted SElQ (final concentrations: 0.1, 1 and 10 µg/ml) were added into the upper chamber of the insert (0.4 µm in pore diameter, Corning, USA), and PBMCs alone were included as a control group. After incubation for 48 h at 37 °C with 5% CO_2, the insert was removed, and the tumour cells were stained using LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen, USA) and imaged under an inverted fluorescence microscope (Leica, Germany).

Additionally, we directly observed that SElQ-treated PBMCs could kill A431 cells under a live cell imaging system. Briefly, A431 cells (2×10^5 cells/1 ml) were seeded in glass-bottom cell culture dishes (Φ20 mm, NEST, China) for 12 h, and PBMCs (4×10^6 cells/1 ml) stimulated with SElQ (final concentrations: 1 µg/ml) were then added into the dish as the experimental group, unstimulated PBMCs served as the control group. After incubation for 24 h at 37 °C with 5% CO_2, the cells were transferred to a live cell imaging system (ACEA, San Diego, CA, USA) and observed for 6 h.

In vivo antitumour activity assays

To establish the tumour model, 2×10^6 murine breast cancer cells (EMT-6) were subcutaneously injected at day 0 into the right axilla of female BALB/c mice to induce the formation of tumours. To examine the antitumour effect of SElQ, the tumour-bearing mice were randomly divided into two groups (four mice per group) on day 1: control group (equal volume of PBS, i.v. into the tail vein, at 2-day intervals) and experimental group (SElQ, 30 µg/mouse, i.v. into the tail vein, at 2-day intervals). All the mice received PBS or SElQ on days 1, 3, 5, 7 and 9. During the experiment, the tumour volume was observed every 2 days beginning on day 5 and calculated using the conventional formula: tumour volume (mm^3) = the longest diameter × the shortest diameter^2/2. The tumour growth inhibition rate was then calculated using the following equation: tumour growth inhibition = (tumour volume of the control group − tumour volume of the SElQ treatment group)/tumour volume of the control group × 100%. At the end of the experiment (day 15), all the mice were killed, and the tumour tissue, spleen and liver were immediately dissected and weighed. Then, the spleen and liver indexes (SI and LI, respectively) were then calculated using the following equation: weight of spleen or liver/weight of mouse. Additionally, the body weight variety index (BWVI) was calculated using the equation: (body weight on testing day − body weight on day 0)/(body weight on day 0), and then mean food consumption (MFC) was calculated as: (food consumption of each group on testing day)/(the number of mice in the cage).

Statistical analyses

The results are presented as the means ± SDs. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software). Pairwise comparisons were performed by two-tailed unpaired Student's *t* test. Statistical comparisons between groups were performed by one-way ANOVAs followed by Dunnett's test. $P < 0.05$ was considered statistically significant.

Results

Murine splenic lymphocyte and human PBMC proliferation assays

The proliferation of murine splenic lymphocytes and human PBMCs stimulated with SElQ was determined by the MTT assay. As shown in Fig. 1a, compared with the negative control (BSA), SElQ significantly stimulated the proliferation of murine splenic lymphocytes in a dose-dependent manner. In accordance with these results, SElQ also stimulated the proliferation of human PBMCs ($P < 0.05$), but the high concentration of SElQ (10 µg/ml) was likely to induce immunosuppression and inhibit the proliferation of human PBMCs (Fig. 3a). The above-described results demonstrated the proliferative activity of SElQ.

T cell-stimulating potency of SElQ

The absolute numbers of murine splenic lymphocytes after stimulation with SElQ were calculated, and the percentages of $CD4^+$ and $CD8^+$ T cells were detected by flow cytometry. As shown in Fig. 2, compared with the unstimulated group, SElQ stimulation significantly promoted the proliferation of murine splenic lymphocytes ($P < 0.05$), and the percentages and absolute numbers of $CD4^+$ ($P < 0.01$) and $CD8^+$ ($P < 0.05$) T cells, particularly those of $CD4^+$ T cells, were significantly upregulated.

Cytokine release in vitro and in vivo

The concentrations of IL-2 and IFN-γ in the supernatants of murine splenic lymphocytes incubated with different concentrations of SElQ in vitro for 48 h were detected by ELISAs. As shown in Fig. 1c, d, the secretion of IL-2 and IFN-γ was significantly increased by SElQ in

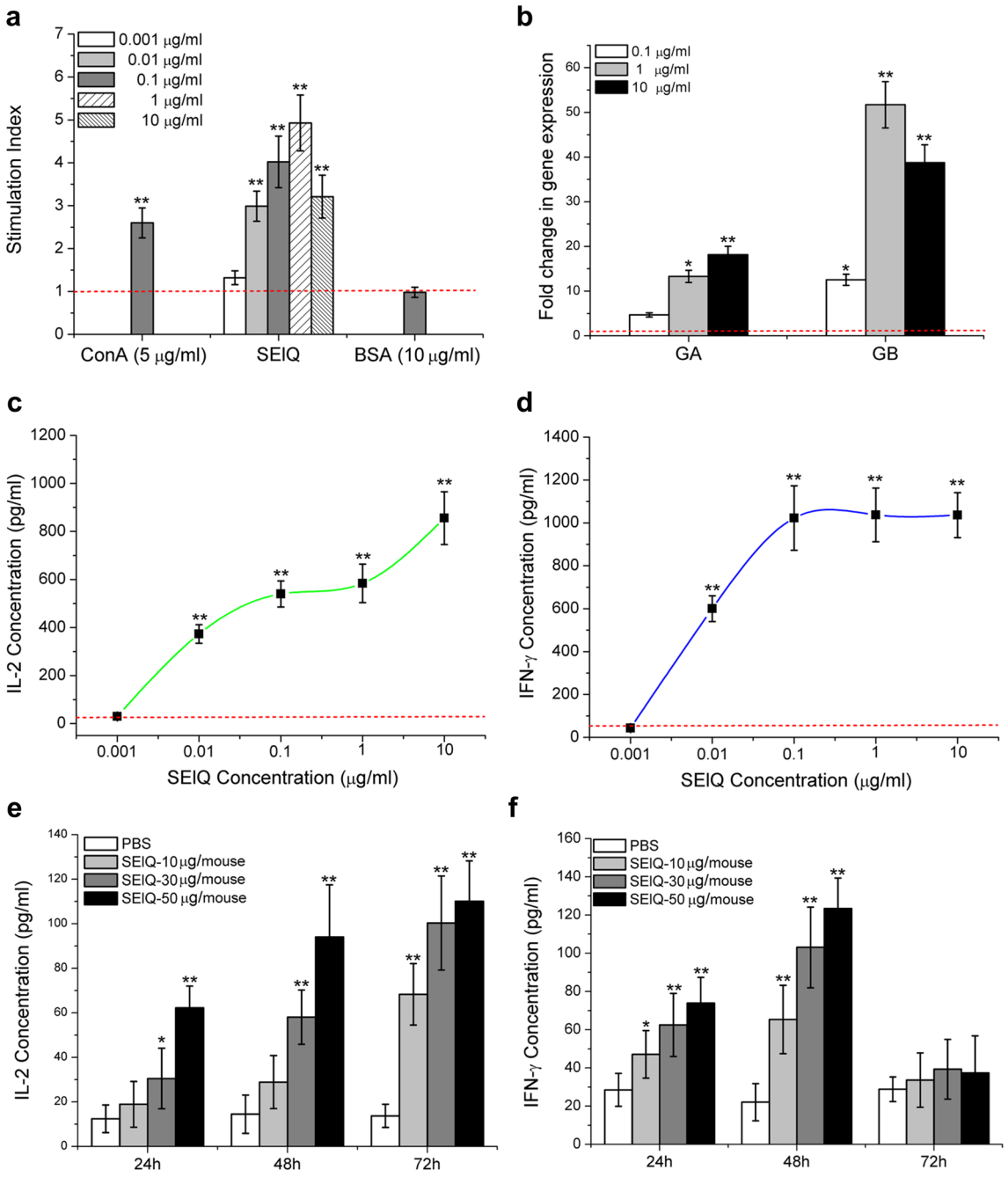

Fig. 1 Effect of SEIQ on murine splenic lymphocytes. **a** Murine splenic lymphocytes were incubated with different concentrations of SEIQ for 48 h, and their proliferation capacity was determined by the MTT assay ($n = 5$). The red dashed line shows the PI of the negative control group (BSA treatment). **b** Fold changes in the transcription of genes encoding T cell-associated effector molecules in murine splenic lymphocytes induced by different concentrations of SEIQ. The results are presented as fold changes relative to the control, which was set to a value of 1 (red dashed line). **c**, **d** Cytokine release from murine splenic lymphocytes treated with different concentrations of SEIQ for 48 h. The concentrations of IL-2 and IFN-γ in the supernatant were measured using ELISA kits, and the red dashed line indicates the concentrations of IL-2 and IFN-γ obtained for the control group. **e**, **f** The concentrations of IL-2 and IFN-γ in the serum of mice treated with different concentrations of SEIQ for 24 h, 48 h and 72 h were examined using ELISA kits. An equal amount of PBS treatment was used as the control treatment. The data shown are representative of at least three separate experiments and are presented as the mean ± SD, and significant differences were detected by one-way ANOVA followed by Dunnett's test; $^{*}P < 0.05$; $^{**}P < 0.01$

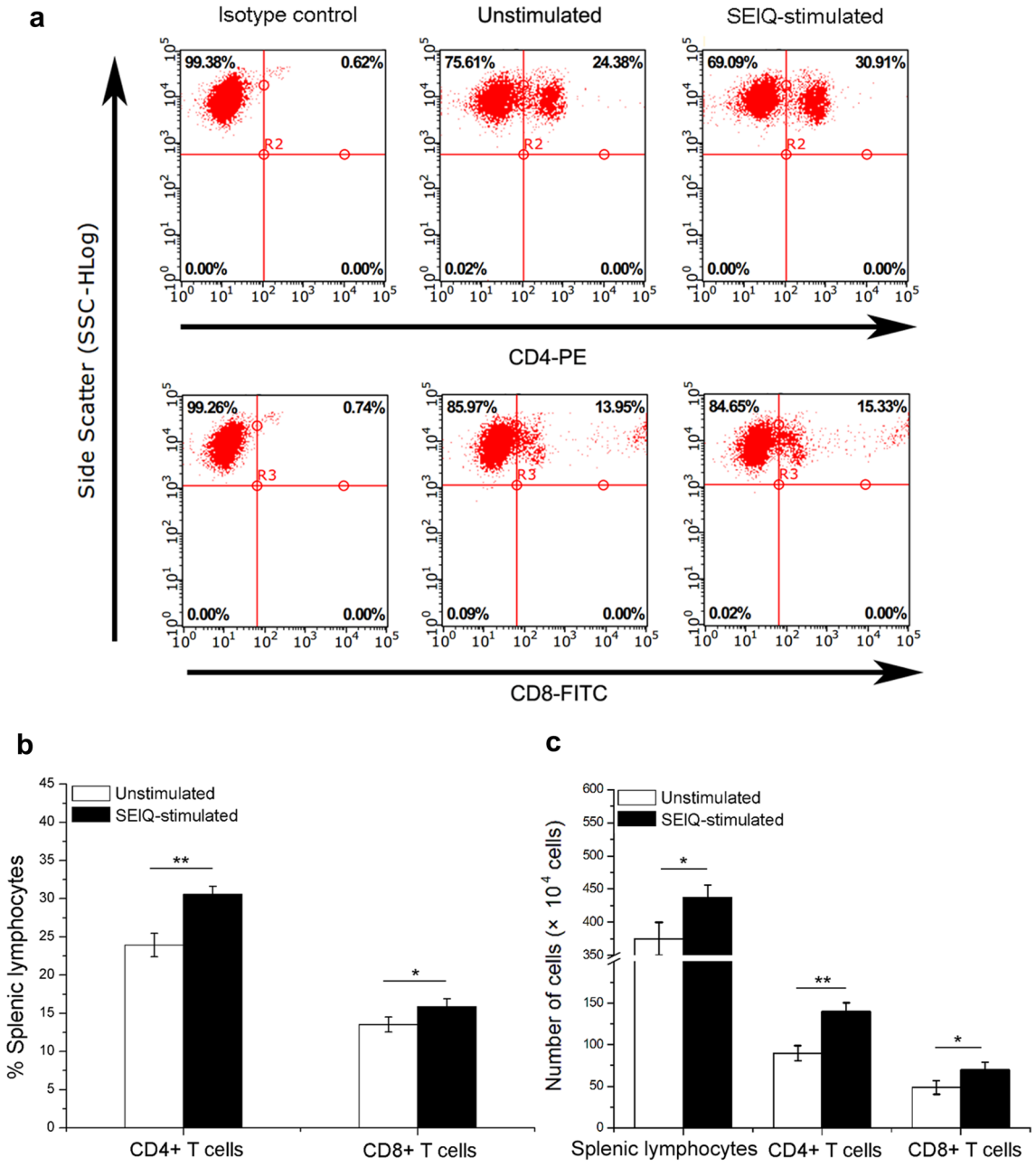

Fig. 2 Proliferation of $CD4^+$ and $CD8^+$ T cells stimulated by SEIQ. **a** Flow cytometry was used to examine the percentages of $CD4^+$ and $CD8^+$ T cells in population of murine splenic lymphocytes stimulated with SEIQ (1 μg/mL). **b**, **c** Percentages and absolute numbers of $CD4^+$ and $CD8^+$ T cells in population of SEIQ-stimulated and unstimulated splenic lymphocytes. The data shown are representative of at least three separate experiments and are presented as the mean ± SD, and significant differences were detected by unpaired Student's *t* test. $^*P < 0.05$; $^{**}P < 0.01$

a dose-dependent manner. Simultaneously, 0.01 μg/ml of SEIQ was sufficient to activate murine splenic lymphocytes in vitro and significantly increase the secretion of IL-2 and IFN-γ. Furthermore, the qRT-PCR results (Fig. 1b) indicated that in addition to increasing the expression of IL-2 and IFN-γ at the protein level, incubation with SEIQ significantly dose-dependently promoted the gene expression of granzyme A and granzyme B in a dose-dependent manner, which are markers of cytotoxic T lymphocytes. In accordance with the release of cytokines observed in the in vitro experiment, the expression of IL-2 and IFN-γ in the serum of mice that received SEIQ by i.v. gradually increased over time in a dose-dependent manner

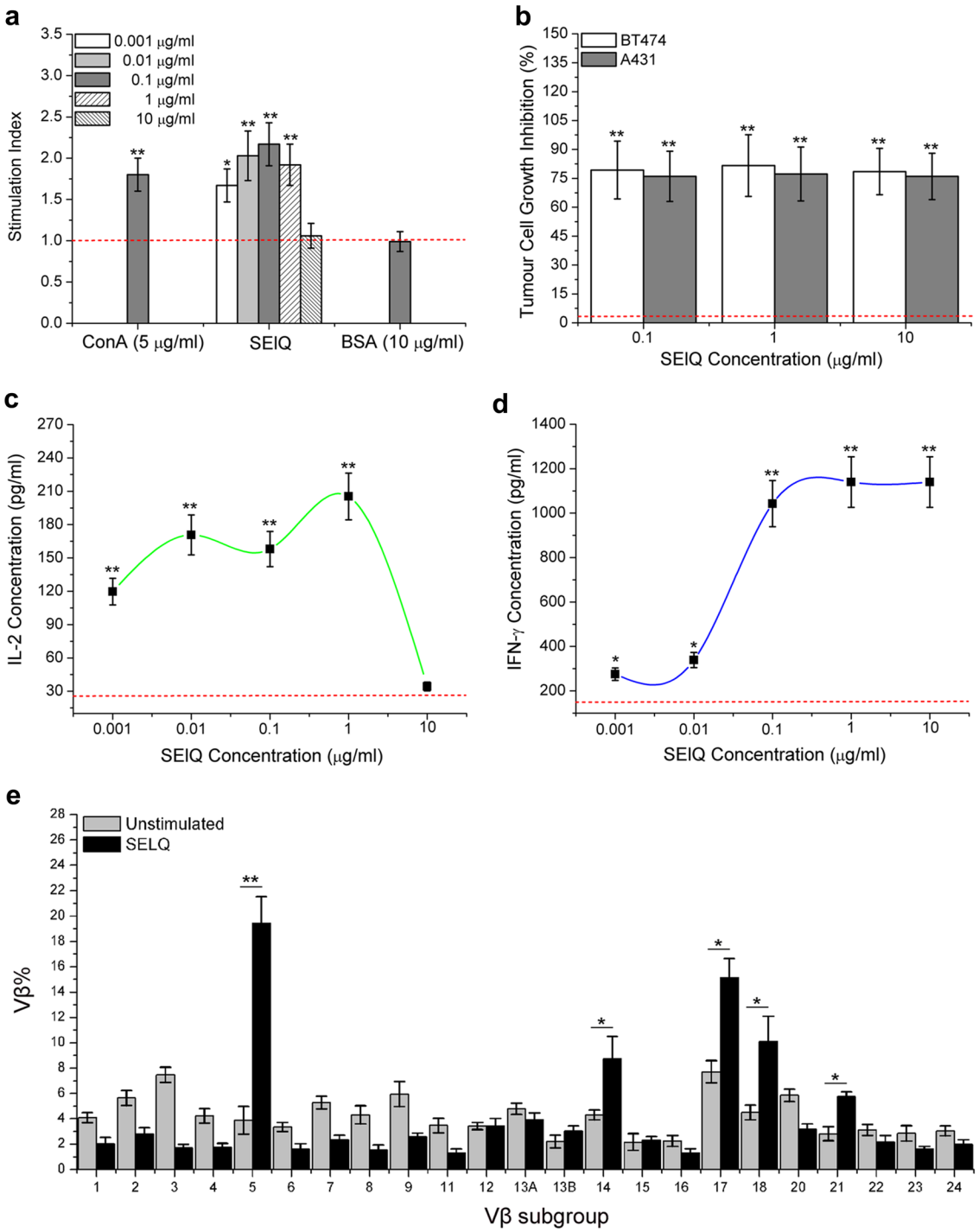

Fig. 3 Effect of SEIQ on human PBMCs. **a** PBMCs were incubated with different concentrations of SEIQ for 48 h, and the proliferation of PBMCs was then determined by the MTT assay ($n = 5$). The red dashed line indicates the PI of the negative control group (BSA treatment). **b** PBMCs were co-cultured with tumour cells (BT474 and A431) and treated with different concentrations of SEIQ. The growth inhibition of tumour cells was calculated by the MTT assay. The red dashed line indicates the inhibition of the negative control group (PBS treatment). **c**, **d** Cytokine release from PBMCs treated with different concentrations of SEIQ for 48 h. The concentrations of IL-2 and IFN-γ in the supernatant were measured using ELISA kits. The red dashed line indicates the concentration of IL-2 and IFN-γ obtained for the control group. **e** cDNA was harvested from PBMCs cultured with SEIQ (1 μg/mL) for 48 h using conventional method and the *V*β profiles were then detected by qRT-PCR as previously reported. Untreated PBMCs served as a negative control. The data shown are representative of at least three separate experiments and are presented as the mean ± SD. Significant differences were detected by one-way ANOVA followed by Dunnett's test (**a**–**d**) and unpaired Student's *t* test (**e**). $^{*}P < 0.05$; $^{**}P < 0.01$

(Fig. 1e–f), but the expression of IFN-γ in the serum of SElQ-treated mice was significantly decreased at 72 h.

Additionally, similar to the results found for murine splenic lymphocytes stimulated with SElQ, human PBMCs incubated with different concentrations of SElQ for 48 h secreted high levels of IL-2 and IFN-γ into the supernatant in a dose-dependent manner, as shown in Fig. 3c, d. However, the secretion of IL-2 from PBMCs was significantly inhibited by the high concentration of SElQ (10 μg/ml), whereas the high concentration of SElQ (10 μg/ml) had no influence on the secretion of IFN-γ.

Expansion of human TCR VβT cells with SElQ stimulation

Based on a well-established method, the selective expansion of T cells bearing certain *V*β subgroups after stimulation with SElQ was examined by qRT-PCR. As shown in Fig. 3e, compared with the control group, the T cells bearing TCR Vβ5 ($P < 0.01$), Vβ14 ($P < 0.05$), Vβ17 ($P < 0.05$), Vβ18 ($P < 0.05$) and Vβ21 ($P < 0.05$) were significantly activated by SElQ stimulation, and the most notable results were found for T cells bearing TCR Vβ5.

In vitro tumour growth inhibition assay

The effects of growth inhibition on BT474 and A431 tumour cells induced by SElQ-activated PBMCs were first examined by the MTT assay. As shown in Fig. 3b, SElQ exerted a promising antitumour effect on two types of human-derived cancer cells in vitro ($P < 0.01$) when compared with the negative control (PBS), and the tumour cell growth inhibition rate reached 80%. Subsequently, A431 cells that were co-cultured with SElQ-stimulated PBMCs under non-contact conditions showed significant cell death, as demonstrated through DEAD/LIVE staining, and most of tumour cells in the culture medium were dead (Fig. 4a). In addition to the antitumour effect of the cytokine cocktail released from SElQ-treated PBMCs, under live cell imaging system we also directly observed that SElQ-activated PBMCs were capable of killing A431 cells (Fig. 4b, Video S1-Unstimulated and Video S2-SElQ stimulated).

In vivo tumour growth inhibition assay

The in vivo antitumour activity of SElQ was further evaluated in mice bearing EMT-6 tumours. At the end of the experiment, the tumour tissues were carefully isolated from the killed mice, as shown in Fig. 5b, c, and further measurement revealed that the tumour weight was significantly decreased in the mice treated with SElQ (30 μg/mouse). Moreover, the tumour volume showed significant inhibition at all tested time points (Fig. 5a), and the inhibition rate was approximately 30%. Additionally, the SI, TI, MFC and BWVI of the experimental mice were examined to evaluate the toxicity induced by the SElQ treatment, and no significant differences in the above indexes were found between the experimental mice and the PBS-treated mice (Fig. 5d–f). The above results indicated that SElQ has promising antitumour activity and low toxicity in vivo.

Discussion

To date, because SEs are capable of inducing an enormous expansion of T lymphocytes bearing certain TCR Vβ regions and thereby give rise to strong T cell-mediated immune activation and cytokine release, the studies on SEs have mainly focused on the interaction of SEs with lymphocytes and the signal transduction pathways related to their activation of the immune response [15–18]. The strong immune activation induced by SEs could trigger food poisoning in healthy individuals, but tumour patients are likely to benefit from this immune activation, which indicates that SE treatment could induce a promising antitumour effect. At present, a considerable number of studies have demonstrated the tumour-therapeutic potential of SEs and confirmed their strong antitumour effect in vitro and in vivo after SE treatment [19]. In China, SEA, SEB, and SEC2 have been used as supplementary pharmaceutical ingredients for tumour treatment. As classic superantigens, SEA can effectively inhibit the growth of melanoma; additionally, SEB can activate T cells to inhibit the growth of or directly kill bladder tumour cells both in vitro and in vivo, and SEC2 has been used as a supplementary medicine for tumour treatment over the past 2 decades [8, 20, 21].

However, the side effects caused by classical SE treatment, such as fever, emesis, and hypertension, seriously limit the application of SEs in the clinic [22]. Additionally, the toxicity of SEs has been positively correlated with their superantigen activity; therefore, a screening for new enterotoxins with efficient superantigen activity and low toxicity would provide support for the further development of new antitumour agents. Fortunately, in 2004, the International Nomenclature Committee named SEs that either lack emetic activity or have not been tested as staphylococcal enterotoxins-like (SEls) [10]. Although the emetic potentials of the primarily recognized SEls, SElK-Q, has been confirmed in cynomolgus monkeys, this potential can be neglected compared with that of classical SEs and can be tolerated by tumour patients [23].

SElQ, which was first identified in 2002, can elicit strong stimulatory effects on the proliferation and cytotoxicity of

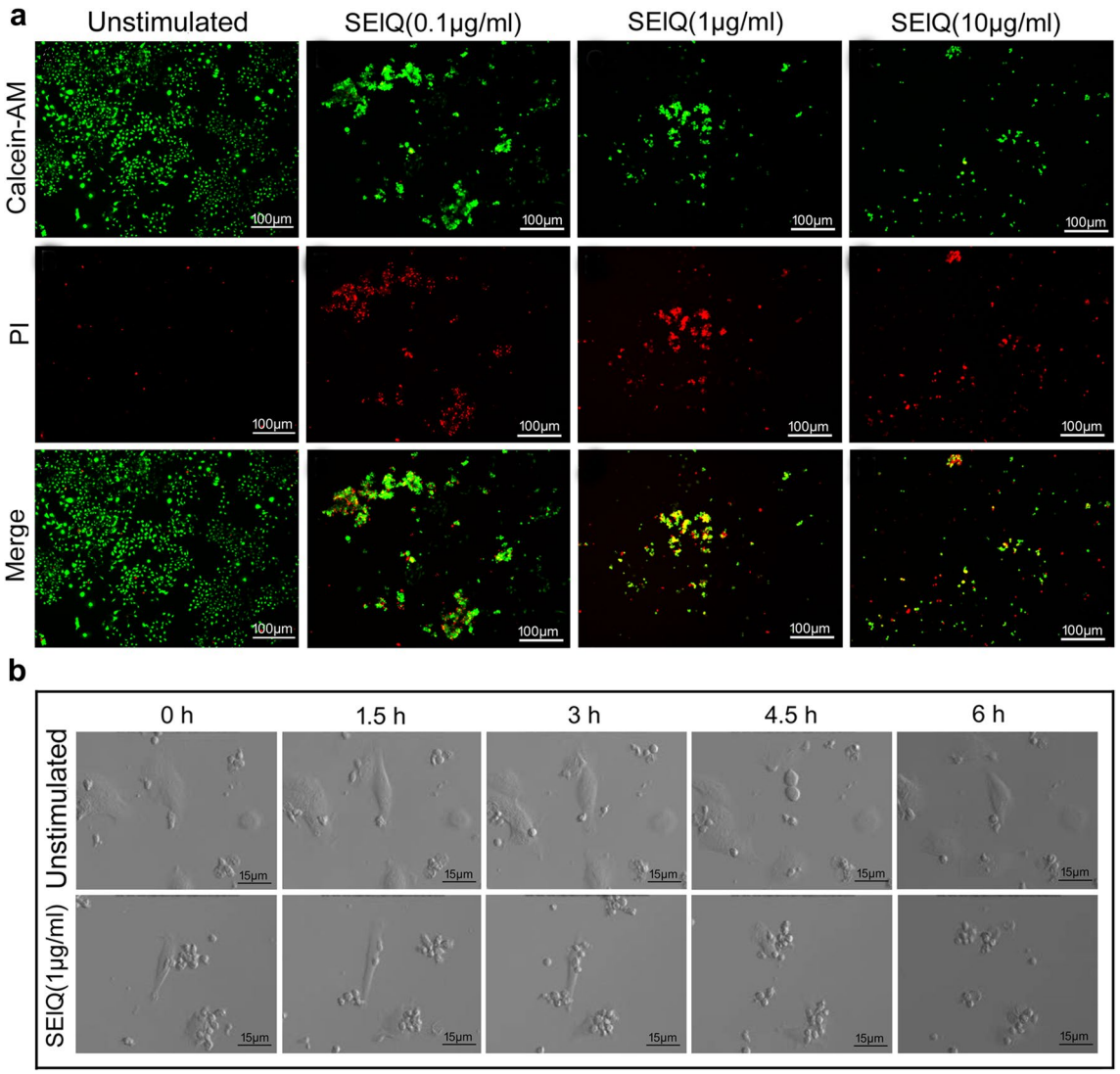

Fig. 4 Mechanism of SElQ-induced antitumour activity in vitro. **a** A431 cells co-cultured with SElQ-stimulated PBMCs for 48 h under non-contact conditions showed significant cell death, as demonstrated by DEAD/LIVE staining, and most of tumour cells in the culture medium were dead. **b** SElQ-activated PBMCs were capable of killing A431 cells, as observed using a live cell imaging system

murine splenic lymphocytes in vitro and does not exert lethal effects in rabbits, as demonstrated using the mini-osmotic pump model, whereas SElK, which was identified adjacent to SElQ, is lethal [24, 25]. Subsequent studies also demonstrated that the SElQ gene could be cloned from the production strain used for staphylococcin injection, which has been commonly used in combined cancer treatments to enhance the systemic immune response [26]. The recent confirmation of the thermostability and digestive enzyme sensitivity of SElQ suggests that this SEl is sufficiently stable for application in cancer treatment [27]. However, the in vivo antitumour activity of SElQ had not been reported prior to this study.

Therefore, in this study, we successfully constructed pET28a-SElQ and obtained high-purity SElQ protein using Ni^{+}-affinity magnetic beads**,** which suggests the feasibility of the mass production of SElQ. Subsequent experiments on the SElQ-induced activation of lymphocytes confirmed that both mouse splenic lymphocytes and human PBMCs can be activated by SElQ. In addition, a low concentration of SElQ (1 ng/ml) significantly stimulated the proliferation of human PBMCs and the secretion of IL-2 and IFN-γ into the supernatant but had no effect on mouse-derived lymphocytes, which indicated that human PBMCs were more sensitive to SElQ stimulation than mouse-derived lymphocytes. However, a high concentration of SElQ (10 μg/ml) more easily induced immunosuppression by inhibiting the proliferation of human PBMCs, and this effect was likely due to the significant decrease in IL-2. In accordance with the cytokine release observed in vitro, the expression of IL-2 and IFN-γ

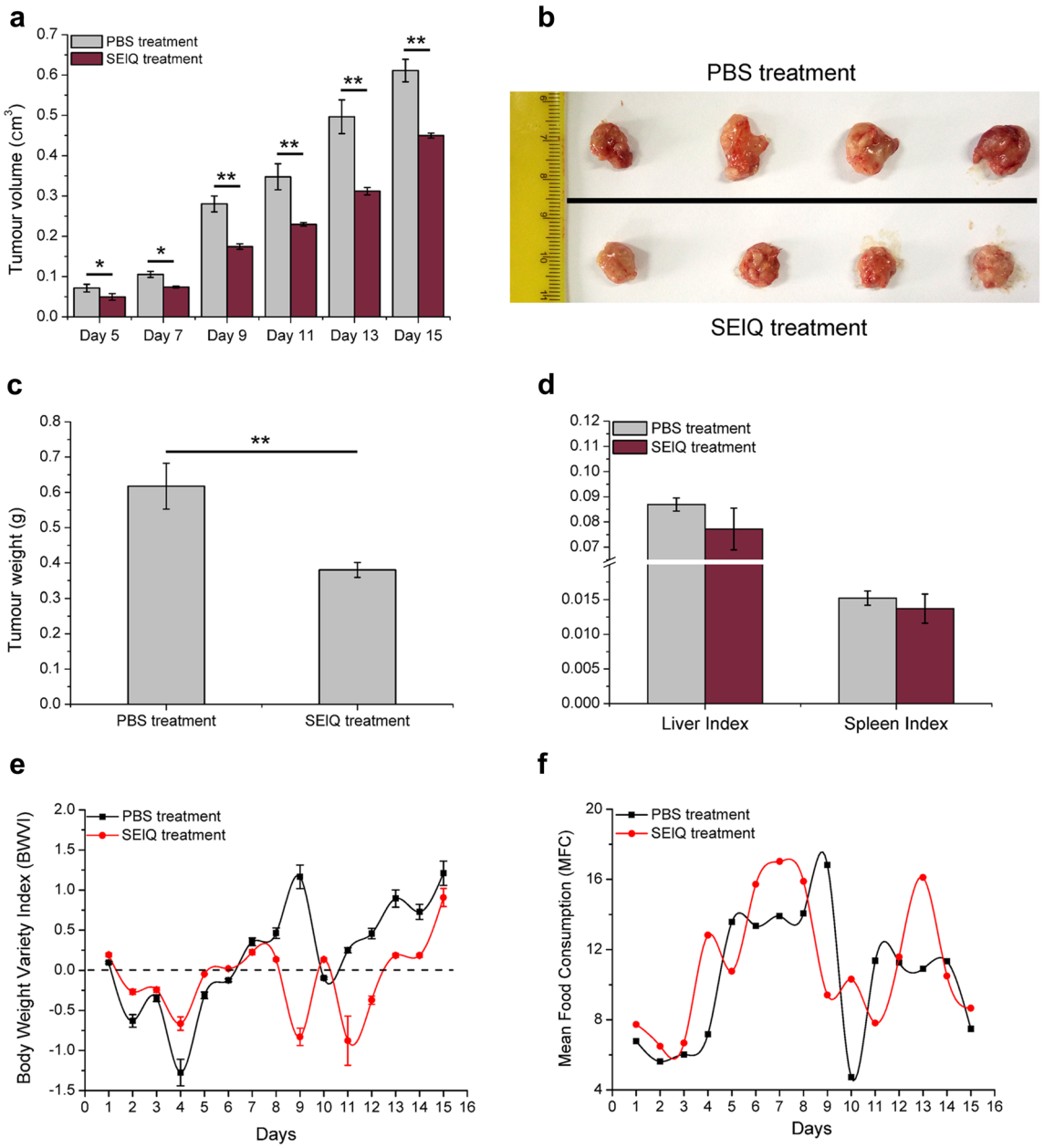

Fig. 5 Antitumour activity of SEIQ in mice bearing tumours. The mice were injected with 2×10^6 murine breast cancer cells (EMT-6) at day 0 and sacrificed at the end of the experiment (day 15). **a** The tumour volumes were calculated at different time points throughout the experiment. **b** The tumour tissues were carefully isolated from the killed mice. The tumour tissue above the line was isolated from the control group (PBS treatment), and that below the line was obtained from the experimental group (SEIQ treatment). The inhibition of tumour growth was quantified. **c** Weights of the tumours isolated from the experimental mice at the end of the experiment (day 15). **d**–**f** The SI, TI, MFC and BWVI of the experimental mice were examined at the end of the experiment (day 15) to evaluate the toxicity induced by SEIQ treatment. The data shown are representative of four independent mice and are presented as the mean ± SD, and significant differences were detected by Student's *t* test. $^*P<0.05$; $^{**}P<0.01$

in the serum of mice treated with SEIQ increased in a dose-dependent manner. However, the expression of IFN-γ was significantly downregulated in the serum of SEIQ-treated mice at 72 h, and this effect is likely due to a self-protective mechanism because a long-lasting high concentration of IFN-γ causes serious side effects in vivo [28].

Furthermore, in the SEIQ-activated mouse splenic lymphocytes, the percentage and absolute number of $CD4^+$ and $CD8^+$ T cells showed significant increases, and this effect was particularly noticeable in $CD4^+$ T cells. Further quantification of the expansion of T cells bearing certain *V*β subgroups after SEIQ stimulation was generally

consistent with previous reports, with only minor variations that could be explained by the differences in the response to SElQ among individuals, differences in techniques (e.g., PCR and flow cytometry), and difference in dosage. In addition to the originally recognized TCR Vβ5 and 21, TCR Vβ14, 17 and 18 were also found to be activated in our study [24]. Additionally, the gene expression of granzyme A and granzyme B was significantly increased in SElQ-activated mouse splenic lymphocytes, which agrees with the increase in $CD8^+$ T cells and suggests an enhancement in the activity of cytotoxic T lymphocytes. The above results suggest that SElQ can elicit substantial antitumour effects both in vitro and in vivo by effectively promoting T cell activation and cytokine release.

As expected, SElQ-activated human PBMCs significantly inhibited the growth of human-derived tumour cells (BT474 and A431) in vitro, and the inhibition rate reached 80%. Subsequent examination further demonstrated that the SElQ-induced antitumour effects were the result of the SElQ-mediated activation of human PBMCs in combination with cytokine cocktail released by these cells. The in vivo antitumour activity of SElQ was then confirmed using a tumour-bearing mouse model with a normal immune system, and although treatment with a low concentration of SElQ (30 μg/mouse) could effectively inhibit tumour growth, the tumour weight and volume continued to grow, which suggested that combined treatments with chemotherapy and/or radiation therapy might be optimal and likely to result in improved antitumour activity in vivo. Additionally, the SI, TI, MFC and BWVI data confirmed that the SElQ treatment has low toxicity and will not cause serious side effects in vivo.

In summary, our results strongly suggest that SElQ can significantly enhance T cell activation and cytokine release and might thus exhibit substantial antitumour activity both in vitro and in vivo. Further studies on SElQ will not only enrich the understanding of SEs but also provide support for its potential application in cancer immunotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The peripheral blood samples used in this study were harvested with the informed consent of the donors, and the experimental procedures performed with the peripheral blood samples were approved by the Ethics Committee of Xinxiang Medical University. Furthermore, the mice handling and experimental procedures were performed with approval from the Animal Care Committee of Xinxiang Medical University in accordance with the guidelines established by the Chinese Council on Animal Care.

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