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Original Article

Toxic effects of seu are different from those of other staphylococcal enterotoxins

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Abstract: Background/Purpose: Staphylococcal enterotoxins (SEs) are soluble extracellular proteins excreted by Staphylococcal bacterial strains, sharing similar structures and virulence. More than 20 genotypes of SEs have been identified, but the toxicity of some new SEs is still unclear. Methods: In this study, we assessed the toxicity effects of six recombinant SEs (rSEA, rSEO, rSEG, rSEK, rSEU and rSEQ) on Balb/c mice by reverse transcription-polymerase chain reaction (RT-PCR)-based methods and enzyme activity detection. Results: Except rSEU, the other five SEs resulted in systemic inflammatory responses with a significant increase of spleen and liver index and decrease of thymus index. SEs enhanced the enzyme activities of liver POD, T-SOD, LDH but reduced the activity of liver GSH-PX. The transcription levels of five cytokines were all down-regulated by rSEA, rSEG and rSEQ at a dose of 20 ng/g, which was coincided with the results of Caspase 3 level. The transcription and expression of IFN-γ, IL-4, IL-6, and TNF-α involved in inflammatory response were significantly up-regulated by rSEs at a low dose (20 ng/mL) except rSEU *in vitro* and *in vivo*. Conclusion: Our results reveals that the rSEA, rSEO, rSEG, rSEK, and rSEQ have cytotoxicity and superantigenicity for Balb/c mice except the rSEU enterotoxin.

Keywords: Staphylococcal enterotoxin, genotype, cytotoxicity, cytokine, enzyme activity

Introduction

As an important food-borne pathogen, *Staphylococcus aureus* presents a significant public health concern. It produces several toxins (e.g., leukotoxins, Panton-Valentine leukocidin, and Exfoliative toxins) and virulence factors including SEs and toxic shock syndrome toxin-1 [1]. The SEs have been known to act as superantigens (SAgs) due to their ability to bind to MHC class II molecules on antigen-presenting cells and stimulate T-cells bearing particularly Vβs on their T-cell receptors [2]. SEs are relatively heat and protease-stable [3], and ingestion of staphylococcal enterotoxins causes emetic responses [4]. The SEs induce acute gastroenteritis in human [5], and cause Kawasaki disease, atopic dermatitis and nasal polyps [6-8].

The SEs have potent effects on the immune system and a pathophysiological role in toxic shock [9]. After the administration of SEs, overproduction of numerous inflammatory mediators results in activation of leukocyte/endothelial cell, and increases endothelial permeability and polymorphonuclear neutrophil (PMN) adhesion and migration. Inflammatory cells in the spleen, liver, and bloodstream produce the first cytokine storm. Cytokines, neutrophil-generated reactive oxygen species (ROS) trigger an acute inflammatory response and induce endothelial damage [10]. After adhesion to endothelium, extravasated inflammatory cells and cytokines initiate and amplify inflammation, which thereby induces tissue damage and end-organ dysfunction [11].

Upon interaction with their natural receptors (MHC class II molecules), SEs activate a large fraction of T cells [12], and induce the uncontrolled and systemic release of pro-inflammatory cytokines, including interleukins (IL-1, IL-6), interferon-gamma (IFN-γ), tumor necrosis factor a (TNF-α) etc. [13-15]. It is believed that the massive release of these pro-inflammatory cytokines, such as TNF-α, is the key step leading to cell apoptosis and organ damage [16, 17].

To date, most of the bacterial enterotoxins are primarily found in *Staphylococcus aureus*. The repertoire of this species is comprised of more than 20 genetic distinct SEs with many strains producing at least one genotype [18]. Several classical types (SEA, SEB, SEC, SED and SEE) have been characterized by their capacity to cause food poisoning [19], however, the new proteins (SEIJ, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, SEIQ, SEIU, SEIV and SEIX) whose amino acid sequences are similar to those classical SEs either do not induce emesis or have not been experimentally demonstrated [20]. Because most se/sel genes are carried on movable genetic elements (MGE), this increasingly amounts to the risk of horizontal genetic material transfer, which can potentially modify the ability of certain staphylococcus strains or species to cause disease or promote the evolution of this important pathogen [21-23]. This high prevalence of se/sels can potentially allow inter- or intra-species transfer in light that many non-pathogenic and pathogenic strains usually co-colonialize on the body mucocutaneous surfaces. Moreover, we revealed a high prevalence rate of se/sel genes (90%) with many carrying multiple genes and identified several previously unreported staphylococcal species from diverse sources, including animals, humans, food sources, and soil samples that were also found to carry se/sel genes (unpublished data). Staphylococcus strains or species widely distributed in the environment may results in severe food safety risks and animal industry challenges. But the toxicity and superantigenicity of some new staphylococcal enterotoxin-like proteins is still unclear. It is important to understand the functionality of these novel SEs and their roles in pathogenesis. In the present study, we produced six novel recombinant SEs (rSEs, i.e., rSEA, rSEO, rSEG, rSEK, rSEU and rSEQ) and use the mice model and some cellular and molecular index to explore their influences on the mice cells *in vivo* and *in vitro.*

Materials and methods

Mouse models and housing conditions

Balb/c mice, breeding stock, were kept in Institute of Experimental Animal Center, National Academy of Medical Science (Tianjin, China). The approval number from Tianjin government authority for use of animals in experiments is SYXK-Jin 2011-0008. All animal experiments were performed under specific pathogen-free conditions and in accordance with institutional, state, and federal guidelines. Animal experiments were performed on 6-week-old mice in compliance with the Tianjin University Institutional Animal Care and Use Committee regulations (TJIACUC).

Production of rSEs

The vectors containing SEs (pET28a-sea, pET28a-seo, pET28a-seg, pET28a-sek, pET28a-seu and pET28a-seq) were constructed in our laboratory. All sequences have been submitted to GenBank under accession numbers GQ859135 (sea), GQ859138 (seg), GQ859139 (sek), GQ859136 (seo), GQ859137 (seu) and ABD21542.1 (seq). The rSEs were expressed in *Escherichia coli* BL21 (DE3) and purified on a Nickel affinity column. We analyzed proteins with SDS-PAGE, and the purified proteins were assayed with Western blot analysis by using staphylococcal enterotoxin anti-chicken whole serum. A natural enterotoxin SEA was purchased from The Institute of Biotechnology, Academy of Military Medical Sciences (AMMS) of China.

Mouse lymphocyte proliferation by MTT assay

The effect of SEs on T cells proliferation was performed as follows. After being isolated from thymus of Balb/c mice, lymphocytes were seeded in 96-well cell plates at 2×10^5 cells per well and maintained in RPMI-1640 (Gibco, USA) supplemented with 10% FCS. Lymphocytes were cultured in a humidified 5% CO_2-95% air incubator at 37°C. Then, lymphocytes were respectively stimulated by rSEA, rSEK, rSEQ, rSEO, rSEU, rSEG, or natural SEA (nSEA) at various doses (10, 20, 40, 80 ng mL^{-1}). Lymphocytes incubated with 10 ng mL^{-1} of ConA acted as positive control, and those incubated with phosphate-buffered saline (PBS) acted as blank control. Four replicates were set for each group with at least three times repeating. After being stimulated for 48 h at 37°C, MTT assay was performed to evaluate the influence of these SEs on cell proliferation as reported previously. Absorbance at 570 nm was measured by a microplate reader. The stimulation index (SI) was calculated by formula: $SI = OD_{570\ experiment\ group}/OD_{570\ blank\ control}$ and statistically analyzed by Student's t-test.

Table 1. Primers of cytokines for Real-time PCR

Gene	GenBank ID	Primer	Annealing Tm (°C)	Product Size (bp)
IL-2	NM_008366.3	5'-GCGGCATGTTCTGGATTTGACT-3'	52.5	136
		5'-CTCATCATCGAATTGGCACTCA-3'		
IL-4	M25892.1	5'-TCACAGCAACGAAGAACACCAC-3'	54.6	155
		5'-GCATCGAAAAGCCCGAAAGAGT-3'		
IL-6	NM_031168.1	5'-ATGGCAATTCTGATTGTATG-3'	49.8	212
		5'-GACTCTGGCTTTGTCTTTCT-3'		
IFN-γ	NM_008337.3	5'-AACTCAAGTGGCATAGATGTGGAAG-3'	54.1	256
		5'-TGTTGACCTCAAACTTGGCAATAC-3'		
TNF-α	BC117057.1	5'-TGAGGTCAATCTGCCCAAGTA-3'	55.7	268
		5'-AGGTCACTGTCCCAGCATCT-3'		
β-actin	NM_007393.3	5'-AGAGGGAAATCGTGCGTGAC-3'	55.9	204
		5'-CACAGGATTCCATACCCAAG-3'		

Mouse viscera index (VI) assay

6-week-old Balb/c mice were randomly divided into eight groups with 10 mice in each. SEs including rSEA, rSEO, rSEU, rSEG, rSEK, rSEQ, natural SEA and PBS buffer (10 mmol/mL, pH 7.4) for blank control were respectively injected to each group of mice at the dose of 10 ng/g through caudal vein, and 10 mice of each group got same treatment for repeat. After 72 h feeding, all the mice were weighed and sacrificed to obtain thymus and spleen. These viscera were also weighed and immediately frozen in liquid nitrogen. Mouse viscera index (VI) was calculated with the formula: VI = Viscera weight/Body weight × 100%. Variance analysis was carried out by repeating in each group. We displayed a fold change of the viscera weight over negative control in the graph with significant conducted by Student t-test. 72 hours after treatment the thymocytes were isolated for total RNA extraction and the cytokine mRNA expression was determined by quantitative PCR. Meanwhile, an ELISA assay was carried out to measure the level of the secreted cytokines in the serum.

Enzyme activity assay

Enzyme Activity (EA) analyses were carried out using POD (peroxidase), SOD (superoxide dismutase), LDH (lactate dehydrogenase), and GSH-PX (glutathione peroxidase) kit (Jiancheng Bioengineering Institution, Nanjing, China) according to manufacturer's instructions.

Thymocyte preparation and total RNA extraction

Whole thymuses were removed from each mouse carefully, parts were saved for the experiment involving apoptosis, and the others were used for RNA extraction.

Fresh thymuses were pushed through sterile 200 mesh screens into centrifuge tube supplemented with RPMI-1640. The resulting suspensions were erythrocyte-depleted over red blood lysing solution (Jiancheng Bioengineering Institution, Nanjing, China), washed, counted and resuspended in RPMI 1640 supplemented with 10% FBS.

The total RNA was extracted using Trizol reagent as follows. 50 mg of the thymuses were ground up in the liquid nitrogen and directly immersed in 750 μL of Trizol, and 250 μL of distilled water and 150 μL of chloroform-isoamylalcohol were added. After mixing, the phases were separated by centrifugation (12000 rpm, 10 min). The aqueous phase was transferred to a new tube and 450 μL of isopropanol was added. After mixing and incubation for 5 min at 4°C, RNA was precipitated by centrifugation (12000 rpm, 10 min). The precipitant was washed twice with 70% ethanol. After the removal of the ethanol and 15-min drying, the RNA was resolved in 20 μL of RNAse-free water and stored at -80°C.

Apoptosis assay

To investigate the apoptosis of T-lymphocyte induced by the stimulation of rSEs, we tested the activity of caspase-3 [24]. The caspase-3 enzyme activity was measured in thymuses tissues collected from mice in the different treatment groups using the Caspase-3 Colorimetric Assay kit (Keygen Institute of Biotech, Nanjing, China) based on the ability of the caspase-3 enzyme to release the yellow chromophore p-nitroaniline (pNA) from the colorimetric sub-

strate (Ac-DEVD-pNA) provided in the CaspACE assay system. Approximately 10^6 isolated T-lymphocyte lysates were prepared for each group and the natural SEA treatment, with PBS as blank control. Relative caspase-3 activity for each sample and samples plus SEs were calculated from the standard curve according to the manufacturer's instructions.

cDNA Synthesis and PCR standards

Primers for amplification of cytokines (IL-2, IL-4, IL-6, TNF-α and IFN-γ) were designed with the Primer Premier 5.0 software (PREMIER Biosoft, CA, USA) and shown in **Table 1**. β-actin previously validated as stable expressed control genes were used as endogenous controls. The isolated RNA was reverse transcribed into cDNA using the cDNA Reverse Transcription kit (Applied Transgen Biotech, Beijing, China) following the manufacturer's instructions. Finally, 5 mL of the cDNA solution was used for a 40-cycle Sybrgreen PCR assay using the SYBR® Premix Ex Taq™ (TaKaRa) with the primers described in **Table 1**.

The cytokine target genes were purified by agarose gel electrophoresis and EasyPure Quick Gel Extraction Kit (Applied Transgen Biotech, Beijing, China) according to the manufacturer's instructions. Then, the purified products were ligated into the pGEM-T easy plasmid (Promega), and transformed into *E.coli* DH5α-competent cells for positive selection. The positive plasmids were confirmed by sequencing and used as positive control.

Quantitative real-time PCR (qPCR)

To analyze the genetic transcription of cytokines (IL-2, IL-4, IL-6, TNF-α and IFN-γ) stimulated by the rSEs, a qPCR assay was performed using Sybrgreen PCR assay as described above. Cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60.3°C-64.2°C for 30 s, and 72°C for 1 min. The real-time PCR data were plotted as the ΔRn fluorescence signal versus the cycle number [25]. The fold change of cytokines both in vivo and in vitro in target cDNA relative to the β-actin endogenous control was determined by:

$$\text{Fold change} = 2^{-\Delta\Delta Ct} \quad (2)$$

Where $\Delta\Delta C_t = (C_{t\ \text{Target x}}-C_{t\ \text{Actain}})_{\text{Sample y}}-(C_{t\ \text{Target x}}-C_{t\ \text{Actain}})_{\text{Control}}$. Target x is the cytokines we detected, Actin is endogenous gene in different samples, Sample y is the SEs treatment, and Control is the negative control.

ELISA assay

Five cytokines IL-2, IL-4, IL-6, TNF-α and IFN-γ in mice sera were determined by ELISA kit to reveal expression levels. According to the instructions of mouse cytokine detection kit (R&D, USA), standard curves were constructed as previously described. Simultaneously, the cytokine levels in supernatants of thymocytes from Balb/c mice treated with 10 ng/g rSEs were measured with this assay.

Statistical analysis

The statistical significance of the results was determined by using the software GraphPad Prism (version 5.0a; San Diego, CA), and is expressed as the mean ± standard error of the mean. Statistical significance was determined by a Student's t-test, two-way ANOVA for multiple comparisons. Probability values less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

Expression and purification of recombinant protein

The target proteins were expressed in *E.coli* BL21 (DE3) cells and subsequently purified by using nickel column. Protein molecular weight of six recombinants was detected by SDS-PAGE and their size is consistent with the expected results as 27.8 kDa, 27.6 kDa, 27.9 kDa, 26.2 kDa, 28.0 kDa, and 25.8 kDa, respectively (**Figure 1A**). Antigenicity of the target proteins was identified by Western blot analysis using anti-SE chicken polyclonal antibodies of SEA (**Figure 1B**).

rSE differ in ability to cause mouse T cell proliferation

To study the ability of six rSEs in simulating T cell proliferation, we tested T cells isolated from Balb/c mouse thymus. Under different doses of rSE (10 ng mL^{-1}, 20 ng mL^{-1}, 40 ng mL^{-1}, and 80 ng mL^{-1}) treatment with natural SEA, conA as positive and PBS as blank control, separately, these cells were evaluated by MTT assay after 48 h post treatment. As shown in **Figure 2**, nat-

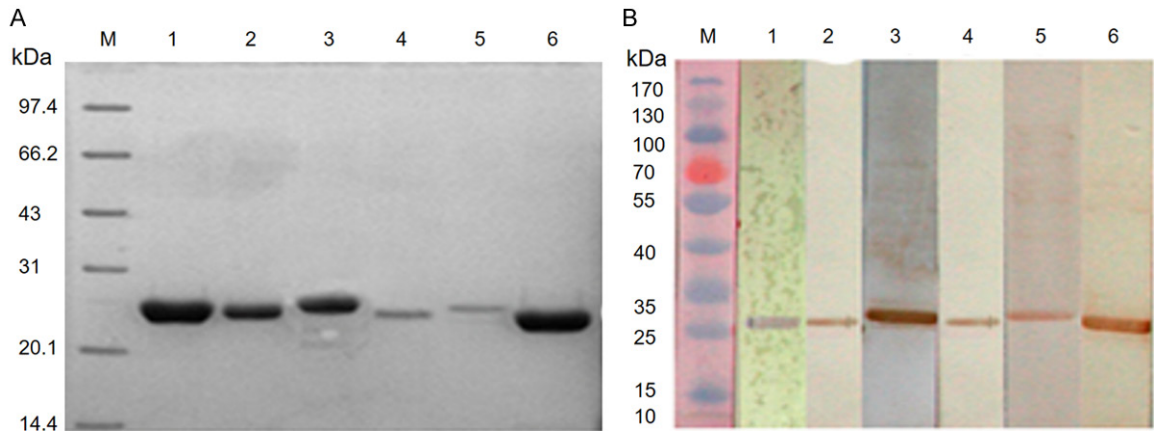


Figure 1. SDS-PAGE and Western-Blot analysis of six rSEs. A. The optimal eluted SEs was analyzed by SDS-PAGE electrophoresis; Lane M represents Protein Marker ranging from 14.4 kDa to 97.4 kDa, Lane 1-6 represent rSEA, rSEO, rSEG, rSEK, rSEU, rSEQ, respectively. B. Western-Blot for the six SEs was analyzed using anti-SE chicken polyclonal antibodies of SEA. Lane M represents Protein Marker ranging from 10 kDa to 170 kDa, Lane 1-6 represent rSEA, rSEO, rSEG, rSEK, rSEU, rSEQ, respectively. rSE, recombinant staphylococcal enterotoxins.

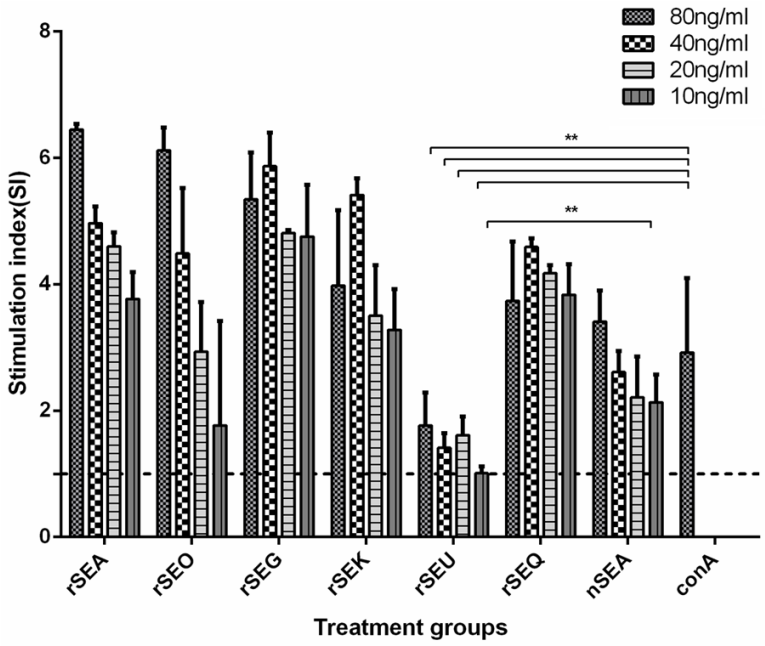


Figure 2. Different doses of rSEs induce distinct T cells proliferation in Balb/c in vitro. MTT assay was carried out in 96 plates after 48 h post treatment at concentrations of 10 ng mL^{-1}, 20 ng mL^{-1}, 40 ng mL^{-1}, 80 ng mL^{-1} covering six rSEs, natural SEA, conA (positive control) and PBS (blank control) . Y axis was the stimulation index, which indicates the value in treatment groups compared to PBS control. SI > 1 indicates an increase in lymphocyte proliferation after treatment. All rSE except rSEU exhibited an extremely significant dose-dependent stimulation activity compared to PBS control ($P < 0.001$), and rSEU has a significant difference ($P < 0.05$) with conA positive control. Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. *indicates significant difference compared with respective control groups. **, $P < 0.01$. rSE, recombinant staphylococcal enterotoxins; SI, Stimulation index.

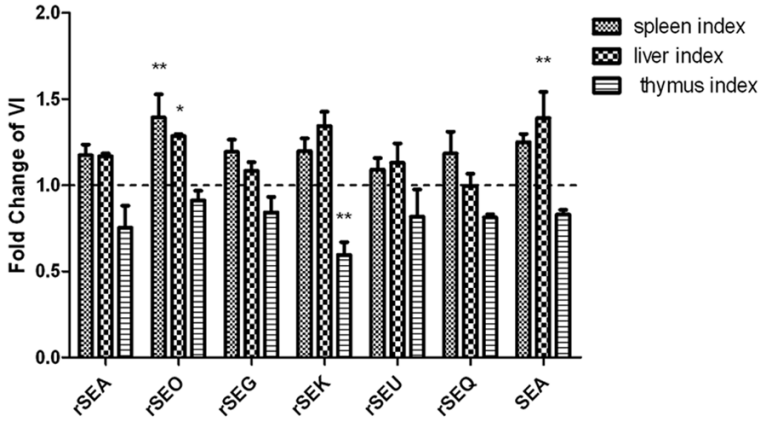
ural SEA, all rSE except rSEU exhibited an extremely significant dose-dependent stimulation activity compared to PBS control ($P < 0.001$), and rSEU has a significant difference ($P < 0.05$) with conA positive control and different SE treatment groups at the same SE stimulation concentration.

Effect of SEs on mice viscera index (VI)

To investigate the impact of SEs on mice spleen, liver and thymus, mice were tail-injected intravenously with 20 ng/g of the six kinds of rSEs and a natural SEA, with PBS blank control. After 72 h, mice were euthanized to obtain the viscera for weight. There was no significant difference from rSEU-stimulated mice and control. However, the administration of other five rSEs, similar with SEA, leads to significant increase of spleen and liver index and decrease of thymus index (T test, $P < 0.05$), compared with PBS control (**Figure 3**).

Effect of SEs on metabolic enzymes of mice liver exogenous compounds

To identify the impact of SEs on the Enzyme Activity (EA) of peroxidase (POD), superoxide



Firgure 3. The effect of high dosage enterotoxins to VI. Fold change was calculated of Mouse Viscera (three columns, spleen, liver and thymus) Index after 72 h high-dose (20 ng/g) SEs (rSEA, rSEO, rSEG, rSEK, rSEU, rSEQ and native SEA) stimulation compared with negative treatment (PBS, not shown in the figure). There was no significant difference from rSEU-stimulated mice and control. However, the administration of other five rSEs and SEA leads to significant increase of spleen and liver index and decrease of thymus index (T test, $P < 0.05$), compared with PBS control. Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. *indicates significant difference compared with respective control groups. *, $P < 0.05$, **, $P < 0.01$, respectively for statistically significant, and very significant differences with respect to conA or nSEA positive treatment. VI, Viscera Index; rSE, recombinant staphylococcal enterotoxins; nSE, nature staphylococcal enterotoxins.

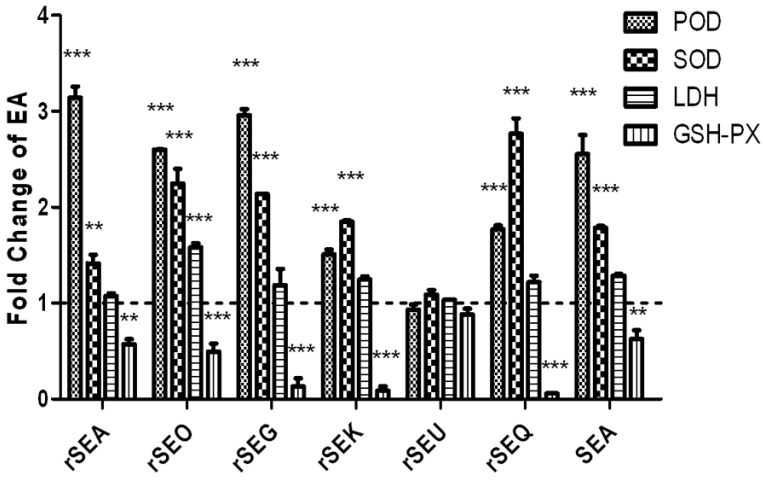


Figure 4. The enzymes activities in liver induced by SEs. Relative fold change of EA (four columns, POD, SOD, LDH and GSH-PX) Index inoculated with rSEs (rSEA, rSEO, rSEG, rSEK, rSEU, rSEQ and native SEA) after 72 h (20 ng/g body weight), compared with PBS control. Stimulation of rSEU has no significant influence on the enzyme activity, the other rSEs and SEA enhance the activities of liver POD, T-SOD and LDH, and reduce the liver GSH-PX activity. Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. **, $P < 0.01$, ***, $P < 0.001$ respectively for very significant and extremely significant differences with respect to PBS treatment. EA, Enzyme Activity; rSE, recombinant staphylococcal enterotoxins; POD, peroxidase; SOD, superoxide dismutase; LDH, lactate dehydrogenase; GSH-PX, glutathione peroxidase.

dismutase (SOD), lactate dehydrogenase (LDH) and glutathione peroxidase (GSH-PX) in mice, we used their respective kit (Jiancheng Bioengineering Institution, Nanjing, China). Mice were tail-injected intravenously with 20 ng/g of SEs, and we obtained liver tissue after three days' raising. Although the stimulation of rSEU has no significant influence on the enzyme activity, the other rSEs, similar to SEA, enhance the activities of liver POD, T-SOD and LDH, and reduce the liver GSH-PX activity (T test, $P < 0.05$) (**Figure 4**).

Effect of SEs on cytokine transcription in vivo

To identify the effect of SEs on the cytokines in vivo mRNA transcriptional level, we inoculated mice with 20 ng/g SEs. TRIZOL method was used to extract the thymus tissue and total RNA, and the extracted RNA was used as a template to obtain cDNA by reverse transcription for real-time PCR mentioned previously. According to the amount of template copies (5 kinds of cytokines) that was calculated, we analyzed the impact of different SEs on the change of cytokines mRNA transcription (**Figure 5**). rSEO and rSEK have similar effects which can raise the level of TNF-α transcription (T test, $P < 0.01$). But the administration of high dose of rSEA, rSEG and rSEQ suppresses the level of cytokines IL-2, IL-4, TNF-α, IFN-γ and IL-6 transcription as natural SEA did (T-test, $P < 0.05$). Except TNF-α, rSEU has no significant effect in other four cytokine transcription (T test, $P > 0.05$).

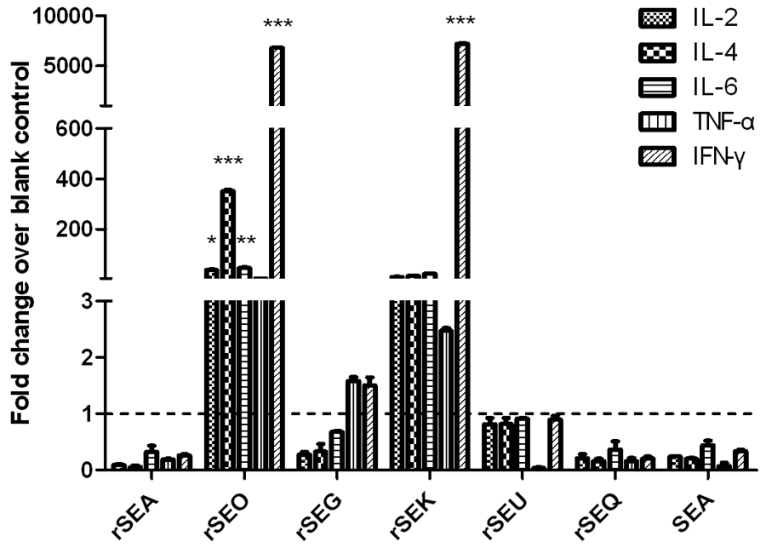


Figure 5. The effect of cytokines transcription induced by high dose enterotoxins. Total RNA from SEs (seven groups in X axis) stimulated Balb/c mouse thymus tissue was applied for relative real-time fluorescentive PCR assay of five cytokines, IL-2, IL-4, IL-6, TNF-α, IFN-γ (five columns). Fold change in Y axis represents cytokines change fold after treatment compared to the PBS control. Fold change > 1 indicates an increase in cytokines transcription after treatment, or is on the opposite. The rSEO and rSEK raise the level of TNF-α transcription (T test, $P < 0.01$). The administration of high dose of rSEA, rSEG, rSEQ and nSEA suppresses the level of cytokines IL-2, IL-4, TNF-α, IFN-γ and IL-6 transcription (T-test, $P < 0.05$). Except TNF-α, rSEU has no significant effect in other four cytokine transcription (T test, $P > 0.05$). Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ respectively for statistically significant, very significant and extremely significant differences with respect to PBS treatment. rSE, recombinant staphylococcal enterotoxins.

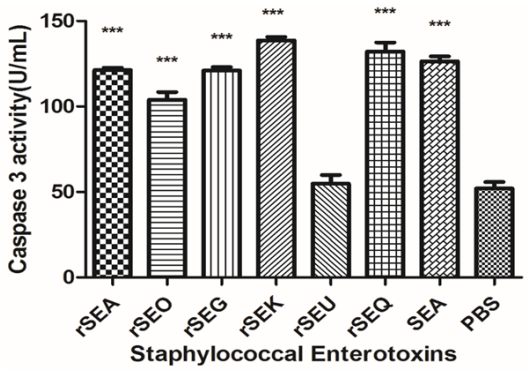


Figure 6. Enzyme activity of Caspase 3 in T-lymphocyte Induced by SEs. T-lymphocyte derived from thymuses showed a marked change to SEs induced apoptosis compared with PBS control ($P < 0.001$), except rSEU (no significant difference). Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. ***, $P < 0.001$ respectively for extremely significant differences with respect to PBS treatment. rSE, recombinant staphylococcal enterotoxins.

T-lymphocyte apoptosis induced by SEs

To determine the apoptosis of T-lymphocyte induced by SEs, we tested the enzyme activity of Caspase-3. As with the natural SEs, the SEs-treated group except rSEU exhibited a significant increase (T test, $P < 0.001$) in the active caspase-3 content to 52±5% of the control value (**Figure 6**). rSEU group alone had no significant effects on active aspase-3 activity (54.82±6% of control). High dose of SEs promoted the Caspase-3 enzyme activity, causing T-lymphocyte apoptosis.

The ability of rSEls to induce cytokine production in vitro

Bacterial enterotoxins, like staphylococcal enterotoxins, via superantigenic stimulation, lead to massive T cell proliferation and secretion of abnormally large amounts of proinflammatory cytokines. In order to consider the stimulating ability of SEK, Q, O, U, A, G to promote cytokines production, the transcriptional profile of a series of designated cytokines was evaluated, including IL-2, IL-4, IL-6, TNF-α and IFN-γ. We treated thymocytes from Balb/c mice with 40 ng/mL rSEs for 72 hours, and then the cytokines in the cell culture supernatants were measured by ELISA. As shown in **Figure 7**, most cytokines measured were significantly increasing in rSEs-treated groups as native SEA-treated group, but in rSEU group, in rSEU-treated cells, no significant difference were detected in five cytokines as PBS control group(T test, $P > 0.05$).

rSEls have a variable effect in stimulating mouse cytokine production in vivo

The immuno-stimulatory effect of six rSEls on the cytokine secretion was also examined *in vivo*. Balb/c mice were inoculated with 20 ng/g body weight of rSEK, rSEQ, rSEA, rSEU, rSEG, rSEO, or natural SEA, PBS group as control, and

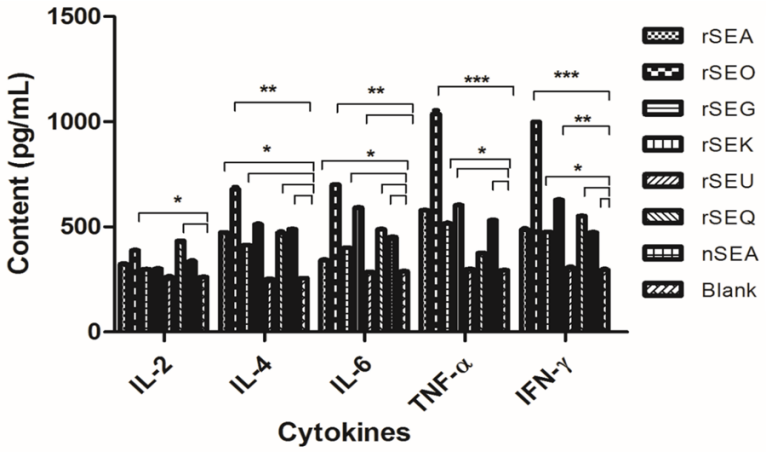


Figure 7. The cytokine content of cell culture supernatant treated with different SEs *in vitro.* Five cytokines (X axis) and their content (pg/mL, Y axis) in supernatant of thymocytes treated by different SEs and PBS control (eight columns) were determined by ELISA kits. Most cytokines measured were significantly increasing in rSEs-treated groups. In rSEU-treated cells, no significant difference were detected in five cytokines as PBS control. Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ respectively for statistically significant, very significant and extremely significant differences with respect to PBS treatment. rSE, recombinant staphylococcal enterotoxins.

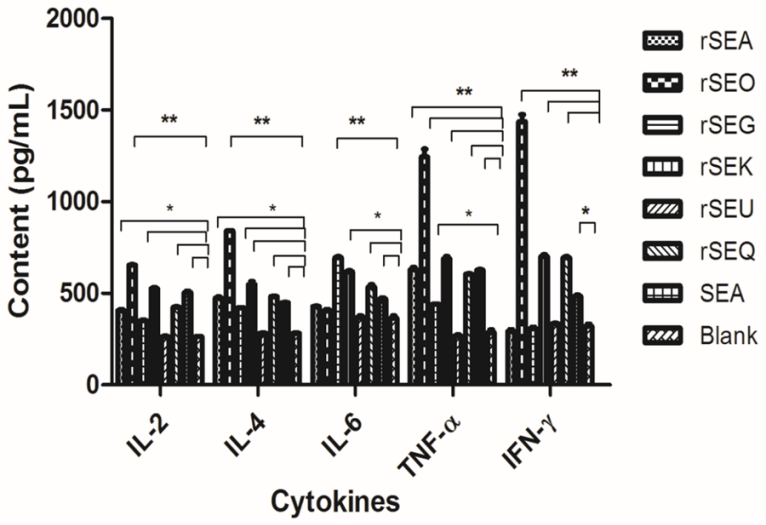


Figure 8. The cytokine content of sera of mice inoculated by different rSEs *in vivo.* Five cytokines (X axis) and their content (pg/mL, Y axis) in sera of Balb/c mice inoculated different SEs and PBS contrl (eight columns) were determined by ELISA kits. rSEU treated mice almost have no effect on five cytokine expression as PBS control (T test, $P > 0.05$). Error bars represent the standard deviations, and statistical significance was determined by using Student's unpaired t-test. *, $P < 0.05$, **, $P < 0.01$, respectively for statistically significant, and extremely significant differences with respect to PBS treatment. rSE, recombinant staphylococcal enterotoxins.

the production of cytokines in the mice sera was measured by ELISA after 72 h. As shown in **Figure 8**, most rSEs induced a vast production of cytokines at protein levels by contrast to the PBS control group. rSEO had a superior effect on the transcription of cytokines such as IFN-γ and TNF-α, the level of which was 2 times higher than that of nSEA control (T test, $P < 0.01$). By contrast, consistent with the *in vitro* assays, rSEU-treated mice almost have no effect on five cytokine expression as PBS control (T test, $P > 0.05$).

Discussion

The pathogenic mechanism induced by Staphylococcus enterotoxin falls into four main categories. (1) Stimulation with SEs produces excess cytokines, chemokines and platelet-activating factors. These agents impel reactive oxygen species (ROS) released from neutrophil, and finally result in tissue edema [26]. Staphylococcal enterotoxins (SEs) induce crosstalk between malignant and benign T cells leading to Stat3-mediated high levels of interleukin-10 (IL-10) production. The malignant activation of the Stat3/IL-10 axis plays a key role in driving the immune dysregulation and severe immunodeficiency that characteristically develops in cutaneous T-cell lymphoma patients [27]. (2) SEs stimulate the inflammatory cells to release a lot of H_2O_2 and OH radicals, which damnify the cells in viscera directly and trigger inflammation cascade reaction to make tissue and organ injured by activating the chemotactic factor further [28]. (3) SEs influenced the activities of metabolic enzymes of exogenous compounds. The enzyme activities can be up- or down-regulated by SEs, which leads to the

dysfunction of organism [29]. (4) SEs can provoke the mechanism of apoptosis [30].

The present study demonstrates that treatment with high doses of SEs significantly increases spleen and liver index and decreases thymus index. Spleen and thymus are the most important immune organ while liver is one of the most important detoxification organs. The administration of high doses of SEs regulates the Viscera Index (VI) by encouraging T-lymphocyte apoptosis in thymus and B cell proliferation in spleen [31]. Meanwhile, it triggers liver tissue edema and phagocyte proliferation because of its digestion and metabolism, which is related with the upregulation of liver index. Additionally, different enterotoxin has various influences on viscera, the novel enterotoxin SEG and SEK indicate more severe impact on viscera than that of rSEU, and this may be related to the structure of SEs.

Staphylococcus enterotoxins induces liver injury by oxidative stress and induction of inflammatory response. SEs stimulate the inflammatory cells to release H_2O_2 and OH radicals, which has recently gained attention as secondary messengers that promote lymphocyte to release chemokines, or the local production of ROS, causing the inflammatory cascade reaction and tissue and organ dysfunction by activating the NF-κB mediating leukocyte adhesion to endothelial cells [32]. The antioxidant system, consisted of cytochrome P450 enzymes, catalase (CAT), superoxide disproportionation enzyme (SOD), glutathione reductase (GSH-R) and glutathione peroxidase (GSH-PX), protects bodies against inflammatory responses. The activity of antioxidant enzyme will go up when exposed to the mild stress to remove oxygen free radicals, and go down if the stress suffered over the body's defense capability, which results in oxygen free radicals accumulation and tissue and organ injury [33-35]. POD, SOD and GSH-PX have been shown to play an important role in the liver detoxification, and LDH is the key enzyme associated with energy metabolism in liver. In this study, mice were stimulated with high doses of SEs to investigate the effects of SEs on the enzyme activities mentioned above.

Staphylococcal enterotoxins (SEs) are superantigenic toxins secreted by Staphylococcus aureus that would cause food poisoning and human diseases [36]. Up to date, more than 20 genotypes of SE and SE-like genes have been discovered. Some classical types of staphylococcal enterotoxin (SEs, SEA to SEE, SEG to SEI) are referred to have the capacity to cause food poisoning [37, 38]. In contrast, although staphylococcal enterotoxin-like proteins (SEl), e.g., SEIJ, SEIK, SEIL, SEIM, SEIN, SEIO, SEIP, SEIQ, SEIS, SEIU, SEIV and SEIT, are homologous and structurally similar to the SEs [39], little is known about the significance of these SEls in strains of staphylococcus bacteria from animal infection, and much of the mechanism of the ability of SEs to induce food poisoning remains unknown. A series of independent studies suggested that most SEs, except staphylococcal enterotoxin-like toxins SEl-S and SEl-T, have been described as superantigens because they can bind as whole molecules directly to MHC class II and TCR β-chain, outside the normal antigen-binding interface that governs peptide MHC recognition [12]. They are well-functioned superantigens defined by their unique ability to systemically alter immune system by affecting T lymphocyte and APCs cytokine production [40].

SEs stimulate T-lymphocyte, macrophage and monocyte to release excessive cytokines to render inflammatory responses, including fever, cytopathogenic effect and dysfunction [11, 41, 42]. It has been shown that the enhanced expression of TNF-α and IFN-γ contributes to the acute and chronic inflammation respectively [41], followed by the up-regulated Viscera Index similar to our observation. Additionally, our study on the construction of the rSEs has indicated the superantigen activities of rSEA, rSEO, rSEQ rSEK and rSEG were stronger than that of the rSEU. The powerful superantigenicity can trigger cell apoptosis by massive expression of inflammatory cytokines, which terminated the transcription of cytokines. Our results of cytokines IL-2, IL-4, TNF-α, IFN-γ and IL-6 are consistent with the conclusion.

In our study, the cytotoxicity and superantigenicity of SEO, SEQ, SEK, SEG and SEA were confirmed in mice model. However, the most surprising finding is that rSEU exhibited very limited cytotoxicity and superantigenic activity if there is any. Most superantigens can bind as whole molecules directly to MHC class II and TCR β-chain, outside the normal antigen-binding interface that governs peptide MHC recog-

nition [43, 44]. However, the binding of some superantigens to MHC proteins can be distinguished by an additional zinc-mediated, higher-affinity binding site on the HLA DR β chain mediated by the conserved H81 histidine residue. This interaction has been reported with SEA, SED, SEE, SEH, and can result in a cross-linking of the MHC on the surface of antigen-presenting cells [45]. The structural difference may partially explain the limited immunostimulatory activity of SEU. It should also be kept in mind that SEs only recognize certain subtypes of TCR Vβ. It is possible that the specific Vβ subtype that SEU recognizes is not present in Balb/c mice. Clearly, it is necessary to test the reactivity of SEU to the specific Vβ subtypes in difference animal models in the future.

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Disclosure of conflict of interest

None.

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