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1 **Superantigenicity Analysis of Staphylococcal Enterotoxins SElK and SElQ in a mouse model**

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1 **Abstract**

2 Staphylococcal enterotoxins (SEs) are superantigenic toxins secreted by *Staphylococcus*
3 *aureus* that is involved in causing food poisoning and human diseases. So far, more than 20
4 genotypes of SE and SE-like proteins (SEls) have been identified. While many SEs have been
5 found to be able to cause food poisoning, it is still largely unknown about the roles of SEIs in food
6 and human health. In this study, we analyzed the superantigenic activity of two new types of
7 recombinant SEIs, SElK and SElQ, in a mouse model. The result shows that the rSEIK and rSEIQ
8 stimulated distinct murine T-lymphocyte proliferation, caused tumefaction in mouse spleen and
9 thymus, SEls induced increase of cytokines (IL-2, IL-4, IL-6, TNF-α, IFN-γ) measured by
10 quantitative PCR and ELISA both *in vitro and in vivo*. The result showed that the rSEIQ displayed
11 stronger superantigenicity than the rSEIK and all caused cytokine storm and inflammatory
12 syndromes. The molecular basis for the difference in the superantigenicity was further analyzed by
13 3D structural modeling. The structural difference (e.g. the critical amino acids of the α3-β8 loop)
14 might partially explain the distinct immune-stimulatory activity of rSElK and rSElQ.

15

16 **Keywords:** Staphylococcal enterotoxin; Superantigencity; T lymphocyte proliferation;
17 Inflammatory cytokines

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1 **Introduction**

2 Food safety issues caused by bacterial enterotoxin food poisoning have increasingly given
3 rise to public concern. Staphylococcal Enterotoxins (SEs), soluble extracellular proteins excreted
4 by *staphylococcus* bacteria, have been in a leading role in plenty of grave food poisoning cases.
5 Among all the *staphylococcus* bacteria, *Staphylococcus aureus* is the major one producing a large
6 variety of enterotoxins, which are responsible for infection and intoxication in human such as
7 acute gastro-enteritis, Kawasaki-like disease, dermatosis, respiratory diseases, presenting specific
8 acute clinical syndromes like food poisoning(1-4). These enterotoxins share certain genetic
9 characteristics, similar structural and biological functions and exhibit superantigenic activity(5-8).
10 The staphylococcal superantigens act as activators to simulate polyclonal T-cell proliferation
11 through crosslinking T cell receptors (TCRs) with major histocompatibility class II (MHCII)
12 molecules on antigen-presenting cells (APCs). The immune response towards SEs was performed
13 by recognizing specific subtypes of Vβ-TCRs with the outer region of MHCII to activate massive
14 TCRs and APCs and simultaneously cause cytokine storm and inflammatory syndromes(7-12).
15 Up to date, more than 20 genotypes of SE and SE-like genes have been discovered. Some
16 classical types of staphylococcal enterotoxin (SEs, SEA to SEE,SEG to SEI,SER to SET)(13, 14)
17 are referred to have the capacity to cause food poisoning(15-18). In contrast, although
18 staphylococcal enterotoxin-like proteins (SEl), e. g, SElJ, SElK, SElL, SElM, SElN, SElO, SElP,
19 SElQ, SElS, SElU, SElV and SElT, are homologous and structurally similar to the SEs(13, 19, 20),
20 little is known about the significance of these SEIs in strains of staphylococcus bacteria from
21 animal infection, and much of the mechanism of the ability of SEs to induce food poisoning
22 remains unknown. Further, their possible role in the development or induction of autoimmune
23 disease has not been described. It has been known that the se/sel genes are carried on movable
24 genetic elements (MGE) such as phages (SEA, SEE, SElP), plasmids (SED, SElJ, SElR), and
25 pathogenicity islands (SaPIs)(3),which are horizontally transferable among staphylococcal strains.
26 This features bring potential crisis to food industry and human health. Thus SEs have risen a wide
27 range of concerns in human health.
28 Staphylococcal Enterotoxins (SEs), as a kind of virulence factors secreted by *S. aureus,* are
29 the major decisive causation of staphylococcal food poisoning and the toxic shock symptoms
30 (TSS)(21, 22). They are well-functioned superantigens defined by their unique ability to

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1 systemically alter immune system by affecting T lymphocyte and APCs cytokine production(23).
2 Orwin *et al.* have examined the biological activities of SElK in the superantigenicity, pyrogenicity,
3 the ability to enhance the lethality in a rabbit model (19, 24). However, little is known about the
4 superantigenicity difference among noval SEs and their roles in food poisoning. Thus, we here
5 aim to explore the superantigenic effect of two new types enterotoxins (SElK and SElQ) in a
6 mouse model. The impact on lymphocyte proliferation and cytokine transcription of them were
7 tested *in vivo* and *in vitro*. Furthermore, the internal molecular relation within superantigenicity
8 and their structures was elucidated, too.

9 **Materials and methods**

10 **Animals**

11 6-wk-old C57BL/6J and BALB/c mice were purchased and maintained in SPF facilities in the
12 Institute of Experimental Animal Center, National Academy of Medical Science (Tianjin, China).
13 Animal experiments were performed in compliance with the regulations of Tianjin University
14 Institutional Animal Care and Use Committee (TJIACUC).

15 **Expression and purification of recombinant SElK, SElQ**

16 Selk (accession no: ABD22279.1) and selq (accession no: ABD21542.1) genes were
17 amplified with Primers：
18 selk1:5’-CC***GGATCC***CAAGGTGATATAGGAATTGA-3’,selk2:5’-AA***CTCGAG***TTATATC
19 GTTTCTTTATAAGA-3’ (669bp);
20 selq1:5’-CA***GGATCC***GATGTAGGGGTAATCAACCTT-3’,selq2:5’-AA***CTCGAG***TTATTC
21 AGTTTTCTCATATGA-3’(660bp). And the two genes were subsequently cloned to pET28α
22 expression vector (25, 26). The positive SEs plasmids (pET28α-SEK, pET28α-SEQ) were
23 constructed and transformed into *E.coli* BL21(DE3) host cell, and then were induced by 0.1mM
24 IPTG at 28°C for 6 hours (27). The recombinant SEs (rSElK, rSElQ) were purified with Ni-NTA
25 purification system (InvitrogenTM, USA) according to the manufacture’s instruction. SAgs were
26 further purified to homogeneity by thin-layer isoelectric focusing as described as Bao [51]. The
27 purified proteins were desalinated and freeze-dried for long-term preservation. Protein quality and
28 bioactivity were analyzed by SDS-PAGE and Western bolt according to the previous protocol(27,

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1 28).

2 **Mouse lymphocyte proliferation by MTT assay**

3 Staphylococcal enterotoxins, rSElK, rSElQ stimulating T cells proliferation (29, 30) were
4 implemented as follows. Lymphocytes were isolated from thymus of BALB/c or C57BL/6J mice,
5 and seeded in 96-well cell plates at 2×10^5 cells/well in 10% FCS-RPMI-1640 (Gibco, USA). The
6 superantigenic effect was explored by treating lymphocytes with various doses (10, 20, 40,
7 80ng/mL) of rSElK, rSElQ and natural SEA, ConA (10ng/mL) as positive control, and PBS buffer
8 as blank control. Four replicates were set for each group with at least three times repeating.
9 Additionally, SEs superantigenic ability was investigated in the presence or without of the A549
10 cells, a human alveolar adenocarcinoma cell line expressing MHCII molecules on cell surface(31,
11 32). A549 cells were seeded at 1×10^5 cells per well with SEs stimulation and the details were
12 summarized in Table 1. Cell incubation was proceeded at 37℃ with 5% CO2, 95% humidity for
13 48h following MTT assay as reported previously(26). Absorbance at 570nm was measured by a
14 microplate reader. The Stimulation index (SI) was calculated by formula: SI= $OD570_{experiment}$
15 $_{group}/OD570_{blank\ control}$ and statistically analyzed by Student's t -test.

16 **Mouse Viscera Index(VI) assay**

17 Six-wk-old BALB/c mice were randomly divided into four groups (SEA, rSElK, rSElQ and
18 PBS) with 10 mice each. The designated SEs were injected to the mice at a dose of 5μg/kg
19 through caudal vein and 10 mice in each group got same treat for repeat. After 72h post treatment,
20 the mice were weighed and sacrificed to obtain thymus and spleen. These viscera were weighed
21 and immediately frozen in liquid nitrogen. Mouse viscera index(VI) was figured out by the
22 formula: VI=viscera weight/body weight x 100%(33).

23 **Relative Real-Time PCR assay**

24 Total RNAs were extracted from SEs stimulated thymus tissue *in vivo* and cultured cells *in*
25 *vitro* by TRIZOL LS (Promega, USA) according to the manufacture′s instruction, and
26 reverse-transcripted by TransScript First-strand cDNA Synthesis kit (TransGen, China). The
27 mRNA transcriptional level of different cytokines (IL-2, IL-4, IL-6, TNF-α and IFN-γ) were
28 detected by relative Real-Time PCR performed on an Applied Biosystems 7500 Real-Time PCR

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1 thermocyler. Then the resulting cDNA template were amplified in a 20 μL PCR reaction
2 system(34) consist of 100 ng cDNA, 10 μL 2×SYBR Green mix (Toyobo, Japan), 20 pmol each
3 primer and DNAase-free water. The Real-time PCR process contained a denaturing step at 95°C
4 for 3 min, followed by 40 cycles including denaturing at 95°C for 30 s, annealing at 49.8° C to
5 55.9°C (table 2) for 60s and then extension for 30s at 70°C, following with melting curve and
6 amplification curve analysis(35). β-actin used as the endogenous gene and mock treated samples
7 as the calibrator were applied for Delta cycle thresholds. The real-time PCR data were plotted as
8 the ΔRn fluorescence signal versus the cycle number(36). Cytokines transcription in target cells
9 were measured by relative quantity against β-actin endogenous control by this formula:
10 $\text{Fold change} = 2^{-\Delta\Delta Ct}$
11 Where$\Delta\Delta C_t = (C_{t\,\text{Target}\,x} - C_{t\,\text{Actin}})_{\text{Sample}\,y} - (C_{t\,\text{Target}\,x} - C_{t\,\text{Actin}})_{\text{Control}}$.

12 **ELISA assay**

13 Five cytokines IL-2, IL-4, IL-6, TNF-α and IFN-γ in mice blood and supernatant from cell
14 culture were determined by ELISA kit to reveal expression levels. According to the instructions of
15 mouse cytokine detection kit (R&D,USA), standard curves were constructed as listed below.
16 Simultaneously, the cytokine levels of SEs-treated cell supernatants were measured by this assay.

17 y = 0.0029x + 0.044, R2 = 0.9882 (IL-2)
18 y = 0.0059x + 0.035, R2 = 0.9863 (IL-4)
19 y = 0.0077x + 0.0395, R2 = 0.9839 (IL-6)
20 y = 0.0014x + 0.0903, R2 = 0.9879 (TNF-α)
21 y = 0.0006x + 0.089, R2 = 0.9866 (IFN-γ)

22 **Homology remodeling analysis**

23 The structure was solved by molecular replacement methods using the structure in PDB as a
24 Model. Based on the homologous templates in PDB database, tertiary structure of SElK (PDB
25 ID:3EA6) and SElQ (PDB ID:2G9H) have been simulated by homology modeling function of
26 Swiss Model (http://swissmodel.expasy.org/). Signal peptide of staphylococcal enterotoxin
27 proteins was analyzed by online analysis software (http://www.cbs.dtu.dk/services/SignalP/).
28 DNAMAN and spdbv 4.0 software were separately applied to analyze amino acid sequences and

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1 spatial structures of the two SEs.

2 **Statistics**

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

7 **Results**

8 **Expression and purification of recombinant SElK and SElQ**

9 To explore superantigencity of rSElk and rSElQ, the corresponding genes were individually
10 cloned into bacterial expression vector PET28α with a his-tag gene sequence linked to 5′ end of
11 the enterotoxin gene to promote protein purification. On the basis of superantigenicity potential,
12 natural SEA was chosen as positive control, whose superantigenic ability has been proved
13 previously(37-39). The two target proteins were expressed in E.coli BL21(DE3) cells and
14 purified by nickel column mentioned in previous method. According to SDS-PAGE
15 electrophoresis, the two proteins revealed uniformity in a single band with the expected molecular
16 weights of 26.2, 25.8 kDa (Fig.1A). Reactogenicity of the two SEls was further verified by
17 western-blot(Fig.1B).

18 **rSElK and rSElQ differ in ability to cause mouse T cell proliferation**

19 To study the ability of recombinant SElK or SElQ in simulating T cell proliferation, we
20 tested T cells isolated from BALB/c or C57BL/6J mouse thymus. Under different doses of
21 rSElK or rSElQ (10ng/ml, 20ng/ml, 40ng/ml, and 80ng/ml) treatment with SEA and PBS as
22 positive and negative controls separately, these cells were evaluated by MTT assay after 48h post
23 treatment. The stimulation index of 3.627 was gotten from ConA positive treatment group. As
24 shown in Fig.2, natural SEA, rSElK and rSElQ exhibited a extremely significant dose-dependent
25 stimulation activity compared to PBS control ($p<0.001$) and a significant difference ($p<0.05$) in
26 different SE treatment groups at the same SE stimulation concentration. SEs can provoke the
27 mechanism of apoptosis(40), high dose (80 ng/mL) SEs may cause T cell apoptosis. The dose of
28 40 ng/mL rSElK caused significantly more proliferation of lymphocytes than 10 ng/mL rSElK in

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1 BALB/c mice ($p<0.001$).

2 TCR expressed on T cells crosslinking with MHC II on antigen presenting cells by
3 superantigen enhance the T cell proliferation. To verify whether co-incubation of T cell with APC
4 cells boost up the numbers of T cell proliferation, we incubated BALB/c or C57BL/6JT mouse
5 thymocytes with a cell line expressing MHC II molecules, A549 cells and stimulated the mixture
6 with 40 ng/mL rSEls. MTT assay was implemented after 48h post treatment. Compared with
7 negative control, the presence of A549 cells significantly increased ($p<0.01$) T cells proliferation
8 under natural SEA, rSElK, and rSElQ treatment (Fig.3). Moreover, addition of A549 into BALB/c
9 mouse thymocytes induced more T cell proliferation than that of C57BL/6JT mouse ($p<0.01$),
10 which indicated a species-diversity in T cell activation. It might imply that TCR-Vβ subsets of
11 T-lymphocytes differ in different mouse strains and various SEs had variable dependence on the
12 Vβ subsets of TCR in the role of promoting lymphocyte proliferation(30).

13 **Effect of recombinant SEls on Mouse Viscera Index(VI)**

14 Mouse viscera index (VI) was also measured to reveal the superantigencity of rSElK and
15 rSElQ *in vivo* in BALB/c mice. The two rSEls were injected into mice at a dose of 5μg/kg. After
16 72h, spleen and thymus index was calculated to reflect the stimulating effect. As expected, rSElK,
17 rSElQ induced evident viscera swelling compared to PBS control and showed a consistent result
18 with the cell stimulation experiment *in vitro*(Fig.4).

19 **The ability of rSEls to induce cytokine production *in vitro***

20 Bacterial enterotoxins, like staphylococcal enterotoxins, via superantigenic stimulation,
21 lead to massive T cell proliferation and secretion of abnormally large amounts of
22 proinflammatory cytokines. In order to consider the stimulating ability of SElK, Q to promote
23 cytokines production, the transcriptional profile of a series of designated cytokines were evaluated,
24 including IL-2, IL-4, IL-6, TNF-α and IFN-γ. We treated thymocytes from BALB/c or
25 C57BL/6JT mouse with 40 ng/mL rSElK or rSElQ in presence or absence of A549 cells for 72
26 hours. Then the cytokine transcription was measured by relative quantitative RT-PCR shown in
27 fig.5A, 5B, 5C. According to the results, rSElK and rSElQ were obvious to raise the level of
28 cytokine transcription, especially in A549 cells co-incubation groups (Fig.5A and 5B). And two

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1 different rSEls caused certain cytokine production to varying degrees. Specifically, the
2 recombinant SEls existed a species-dependent stimulating activity in cytokine transcription,
3 BALB/c mouse interacting with A549 cells triggered larger amount of cytokines
4 expanding(Fig.5A) than that of C57BL/6JT mouse(Fig.5B). In addition, the protein production of
5 these cytokines in the culture supernatants was also tested by ELISA. As shown in figure 6, rSElK
6 and rSElQ both had a comparable way as SEA to incur the five cytokines expanding compared to
7 PBS control.

8 **rSEls vary in stimulating mouse cytokine generation *in vivo***

9 The immuno-stimulatory effect of two rSEls on the cytokine secretion was also
10 examined *in vivo*. BALB/c mice were injected with 5 μg/kg amount of rSElK, rSElQ, SEA or
11 PBS. At 72 hours post treatment, the thymocytes were isolated for total RNA extraction and
12 the cytokine mRNA expression was determined by quantitative PCR. Meanwhile, an ELISA
13 assay was carried out to measure level of the secreted cytokines in the serum. Consistent with
14 the *in vitro* assays, rSElK and rSElQ induced a vast production of cytokines at both mRNA
15 and protein levels by contrast to PBS control (Fig.7A, Fig.7B). As shown in Fig. 7A, rSElK
16 had a superior effect on the transcription of cytokines such as IL-4, TNF-α, the level of which
17 was 2 times higher than that of negative control. By contrast, treatment with PBS did not
18 significantly increase the cytokine transcription. The same was true for the protein production
19 of these cytokines in the sera as measured by ELISA.

20 **Structure homology remodeling of rSEls**

21 At a glance of distinct superantigenic activities related to SElK, SElQ, we tried to explore the
22 reason of different function through homologous structure remodeling, typical SE structures
23 consist of 5 alpha helices and 12 beta sheets (Fig.8A). To search the molecular basis for the
24 SElK and SElQ difference, we first analyzed the MHCII-binding sites on SEls (Fig.8B). The
25 structure of the putative MHC binding site on SElK and SElQ is also structurally homologous to
26 that of SEI, and thus, they likely binds to MHC in a similar fashion as does the latter(41).As we
27 know, SEs harbor two binding sites for MHC II molecules. One is the common sites, located in
28 the N-terminal providing a weak affinity with MHCII molecules(e.g. SEB)(42), while the other is
29 Znic binding sites, located in the C-terminal having a strong affinity with MHCII molecules(e.g.

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1 SEH)(43). The critical residues for znic binding sites contain three amino acids: two histidines
2 and an aspartic acid. This structure has a high affinity, approximately 100 times than that of
3 N-terminal binding sites, making it the main sites for the SEs superantigenicity. It has been well
4 documented that SEA contain binding sites for MHC II molecules, which consist of two
5 histidine residues and an aspartic acid residue (asp227, his187, his225).Referring to our results,
6 SElK and SElQ both possess a zinc-binding sites (Fig.8B and Table 3).

7 **Discussion**

8 SEs have risen a wide range of concerns in human health. Existing epidemiological survey
9 reveals a high prevalence rate (about 95.8% staphylococcal isolates harbored more than 2 se/sel
10 genes) in different staphylococcal species (our unpublished data, not shown here). Specifically,
11 Becker conducted a survey of the superantigen profile of 429 *S. aureus* from human blood
12 samples or nasal discharges, and revealed a high detection rate of sei and seq (55%)(44),
13 which arose our interest to have a deep research into new SEl types like SElK and SElQ.
14 Meanwhile, amounting existing evidence, little is known about the superantigenic activity of
15 SElK, SElQ and their role in food issues involved in public health. Hence we established a set of
16 experiments in mice to evaluate the immune effect of rSElK and rSElQ. First, we measured
17 viscera index of mouse thymus or spleen and conducted T cells proliferation by rSElK, rSElQ
18 treatment compared with the SEA. It indicated a strong stimulating activity for both SEls to trigger
19 T-lymphocyte activation and propagation. Meanwhile, relative quantitative PCR and ELISA
20 analysis of inflammatory cytokines separately revealed a mass of cytokines transcription and
21 expression in post treating samples. In some degree, those attempts partially demonstrated
22 superantigenicity of the two recombinant SEls.

23 Superantigens cross bridge TCRs with MHC II molecules on APCs in a relatively nonspecific
24 manner, inducing highly significant proliferation of T cells and activation of APCs such as
25 macrophages(45-47).Although SEs can promote T-lymphocytes proliferation without APC cells, it
26 can function well with APC participation. IFN-γ was an inducer for MHCII molecules expression
27 on A549 cells, A549 lung carcinoma cells co-incubated with IFN-γ can promote vast amounts of
28 MHCII molecules to incur more T-lymphocytes proliferation than T cells culture alone. It might be
29 associated with MHCII-mediated signal transduction mechanisms(11). At the presence of MHCII

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1 molecules, it is capable of activating tyrosine kinase and membrane phosphoinositide, which
2 eventually leads to expression of inflammatory cytokines and further results in an abundant release
3 of cytokines, followed with IFN-γ, IL-2 stimulating further differentiation and proliferation of
4 T-lymphocytes(11, 22).

5 Previous researches have demonstrated that the SEs superantigens interact with TCR in
6 three modes: First, SEs specifically recognize and bind to certain amino acid residues in CDR2,
7 FR3 regions of TCR, such as TSST(45, 48). Second, SEs specifically recognize spatial structure of
8 CDR2, FR3 rather than amino sequence of Vβ, such as SEB, SEC(46, 49, 50). Third, α3-β8 loop
9 of SEs specifically bonds FR3, FR4 regions of TCR, such as SElK, SEI(50). α3-β8 loop is a
10 hypervariable region which has been found in both SElK and SElQ (Table 3). This loop binds to
11 TCR via two different modes, either directly recognizing certain residues or binding to spatial
12 structure of Vβ,it is critical for the specificity of the interaction of the superantigens with their
13 respective Vβ-TCRs(47). For SEA, three key amino acid residues with 14 aa length of the
14 α3-β8 loop are implicated in TCR binding, in which are two Asp residues and one Ser (Ser172,
15 Asp173, Asp175) forming a strong hydrogen bond with the Vβ amino acids. In this study, the
16 two proteins were at the similar length of α3-β8 loop with 26aa amino acids but possessed
17 different residues. A long length of amino acids (26aa) has a good flexibility and mutable structure
18 for Vβ bonding. For SElK, there are 2 residues (His142,Tyr158) implicated in TCR binding
19 whilst SElQ with Glu188 and Tyr191 (Fig.8C). The Glu residue with a good hydrophilicity can
20 be exposed on molecular surface and is capable of forming hydrogen bonds along with a -OH on
21 phenyl loop. This provides easier accessibility to affine with TCR. In contrast, the predicted
22 residue (His) that is exposed on the surface is only capable of forming intra-molecular
23 hydrogen bonds, making it a lower affinity, and the numerous residues within these SEs-TCRV β
24 complex structure interfaces are likely to contribute significantly to both binding and specificity.

25 In summary, the SElK and SElQ can form signaling complexes with MHC and TCR Vβ
26 molecules. The MHC-SEs-TCR ternary complex have significant functional consequences and can
27 activate SEs-specific T cell signaling pathway to boost cell proliferaton and cause the cytokine
28 storm. Thus,The enhanced activity of SEIK compared to SEIQ and SEA, their structural difference,
29 binding efficiency, variations in their MHC-II binding pocket etc., all those are contributed to their
30 superantigenicity and functions. The structural difference (e.g. the critical amino acids of the

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1 α3-β8 loop) might partially explain the diverse immune-stimulatory activity of SElK and
2 SElQ. In accordance with the diverse proliferation scale of T cells from different mouse series, it
3 should also be kept in mind that SEls only recognize certain subtypes of TCR Vβ. The Vβ
4 subtypes in the bracket are recognized by SElK (5.1, 5.2, 6.7), SElQ (2.1, 5.1, 6.7, 21.3) of human.
5 Proliferation of BALB/c T-lymphocytes was superior to C57BL/6J mouse, which implied that
6 BALB/c mouse with Vβ subtypes of certain types could be recognized by these two SEs just
7 missing in C57BL/6J mice or with more SEs recognition sites in BALB/c T cells than another.
8 Some specific TCR Vβ domain have been shown to be overrepresented in some species and
9 patients with Crohn's disease, a severe inflammatory bowel syndrome(51).

10

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16 The author(s) declared no potential conflicts of interest with respect to the research, authorship,
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18 **Author Contributions**

19 Conceived and designed the experiments: Jinhai Huang. Performed the experiments: Yihe Xia,
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21 Contributed reagents/materials/analysis tools: Jinhai Huang. Wrote the paper: Jinhai Huang.

22

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1 ## Figure legends

2

3 **Figure 1. SDS-PAGE and Western-Blot analysis of rSElK, rSElQ, and natural SEA**,
4 SDS-PAGE electrophoresis of purified proteins rSElK, rSElQ. **B**, Western-Blot analysis of two
5 rSEs. M stands for protein marker.

6

7 **Figure 2. Different doses of SEls induce distinct T cells proliferation in BALB/c in *vitro*.** MTT
8 assay was carried out in 96 plates at concentrations of 10ng/mL, 20ng/mL. 40ng/mL, 80ng/mL
9 covering two rSEls (rSElK, rSElQ), natural SEA (positive control) and negative control PBS.Y
10 axis was the Stimulation Index, which indicates the value in treatment groups compared to PBS
11 control. SI>1 indicates an increase in lymphocyte proliferation after treatment. Error bars
12 represent the standard deviations, and statistical significance was determined by using Student's
13 unpaired t-test.

14

15 **Figure 3. SEs stimulation promotes T cells proliferation at presence of A549 cells.** MTT assay
16 was performed by using three kinds of cell samples subject to rSElK stimulation, rSElQ treatment
17 and non-treatment as the negative control(X axis). Three columns separately represent the samples
18 of C57BL/6J T-lymphocytes/A549 cells, BalB/C T-lymphocytes/A549 cells and BaB/C
19 T-lymphocytes. Error bars represented the standard deviations, and statistical significance was
20 determined by using Student's unpaired t-test comparing to PBS treatment. *, $P<0.05$; **, $P<0.01$;
21 ***, $P<0.001$.

22

23 **Figure 4. Low doses of recombinant SEs affect mouse spleen and thymus index.** Fold change
24 was calculated of Mouse Viscera (two columns, spleen and thymus) Index after 72h low-dose
25 (5μg/kg) SEs (rSElK, rSElQ, natural SEA) stimulation compared with negative treatment (PBS).
26 Error bars represent the standard deviations, and statistical significance was determined by using
27 Student's unpaired t-test. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$ respectively for statistically
28 significant, superior significant and the highest significant differences with respect to PBS
29 treatment.

30

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1 **Figure 5. The ability of rSEls to induce cytokine transcription *in vitro*.** A, The change rate of
2 five cytokines transcription in BalB/C T-lymphocytes/A549 cells. B, The effect of five cytokines
3 transcription in C57BL/6J T-lymphocytes/A549 cells. C, The result of five cytokines transcription
4 in BalB/C T-lymphocytes. Total RNA from three kinds of cells treated by SEs(X axis) *in vitro* was
5 applied for relative quantitative real-time PCR assay of five cytokines, IL-2, IL-4, IL-6, TNF-α,
6 and IFN-γ. Fold change(Y axis) is cytokines change fold mentioned in the former. Error bars
7 represent the standard deviations, and statistical significance was determined by using student's
8 unpaired t-test. *, $P<0.05$ for statistically significant differences with respect to PBS treatment.
9
10 **Figure 6. The ability of rSEls to induce cytokine expression *in vitro*.** Culture supernatants from
11 SEs (three groups in X axis) and PBS stimulated BalB/C T cells were applied for ELISA assay of
12 five cytokines, IL-2, IL-4, IL-6, TNF-α, IFN-γ (five columns). Y axis represents cytokines
13 concentration. The Changes of cytokine expressional level were discerned compared to PBS
14 negative control. Error bars represent the standard deviations, and statistical significance was
15 determined by using Student's unpaired t-test. *, $P<0.05$ for statistically significant differences
16 with respect to PBS treatment.
17
18 **Figure 7. rSEls vary in stimulating mouse cytokine generation *in vivo*. A.** Relative real-time
19 fluorescent quantitation of five cytokine transcription by SEs treatment *in vivo*. Total RNA from
20 SEs (three groups in X axis) stimulated BalB/C mouse thymus tissue was applied for relative
21 quantitative real-time PCR of five cytokines, IL-2, IL-4, IL-6, TNF-α, IFN-γ(five columns). Fold
22 change in Y axis represents cytokines change fold after treatment compared to the negative control.
23 Fold change >1 indicates an increase in cytokines transcription after treatment, or is on the
24 opposite. **B.** ELISA for five cytokines expression *in vivo*. Sera from SEs (three groups in X axis)
25 and PBS stimulated BalB/C mouse were applied for ELISA assay of five cytokines, IL-2, IL-4,
26 IL-6, TNF-α, IFN-γ (five columns). Y axis represents cytokines concentration. The changes of
27 cytokine expressional level were discerned compared to PBS negative control. Error bars represent
28 the standard deviations, and statistical significance was determined by using student's unpaired
29 t-test. *, $P<0.05$ for statistically significant differences with respect to PBS treatment.
30

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1

2 **Figure 8. Structure homology modeling of SElK and SElQ.** A. Three-dimensional
3 representation of the two SE structures. The α-helices and β-stands were in rainbow colours from
4 the N-(blue) to C-termini (red), respectively. α-Helices, β-stands as well as the N- and C-termini
5 were labeled. B, Znic MHC-II binding sites of SElK, SElQ. Backbones, side chains of key
6 residues (labelled) were shown as sticks. Red arrows in yellow circles (dashed lines) indicate Znic
7 MHC-II binding sites on key residues which were listed in Table 3. C, α3-β8 loop on TCR binding
8 sites of the two SEs. The loop between α3 and β8 was shown by C-backbones as sticks and
9 colored according to the accessibility ranging in rainbow color from red (high) to blue (low). The
10 key residues of each TCR binding site were labeled in pink dashed circles and detailed in Table 3.
11

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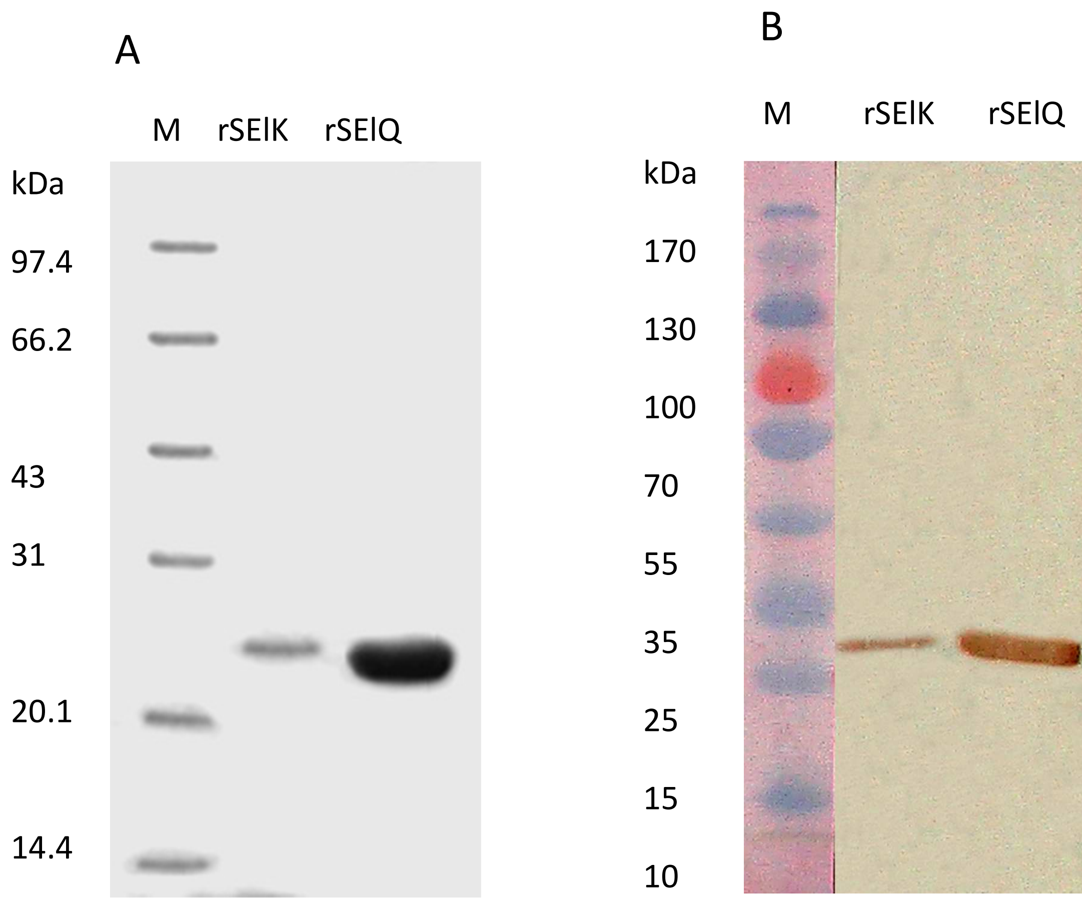

Fig.1

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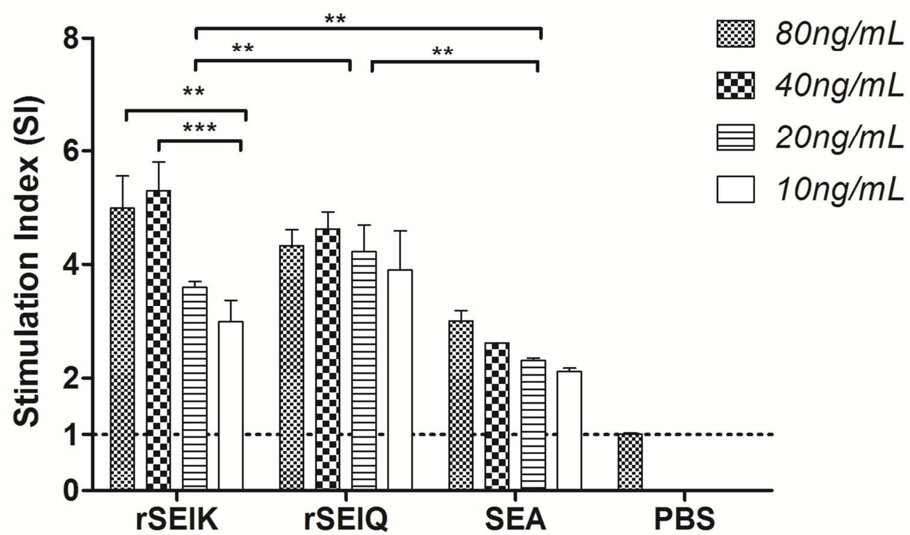

Fig.2

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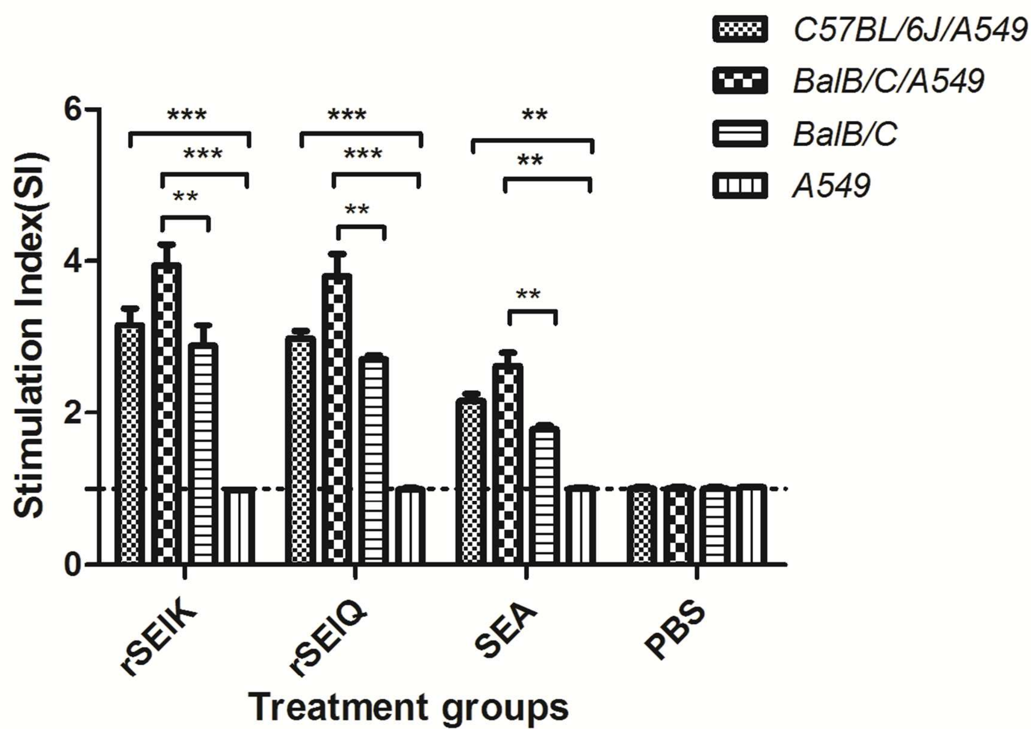

Figure 3

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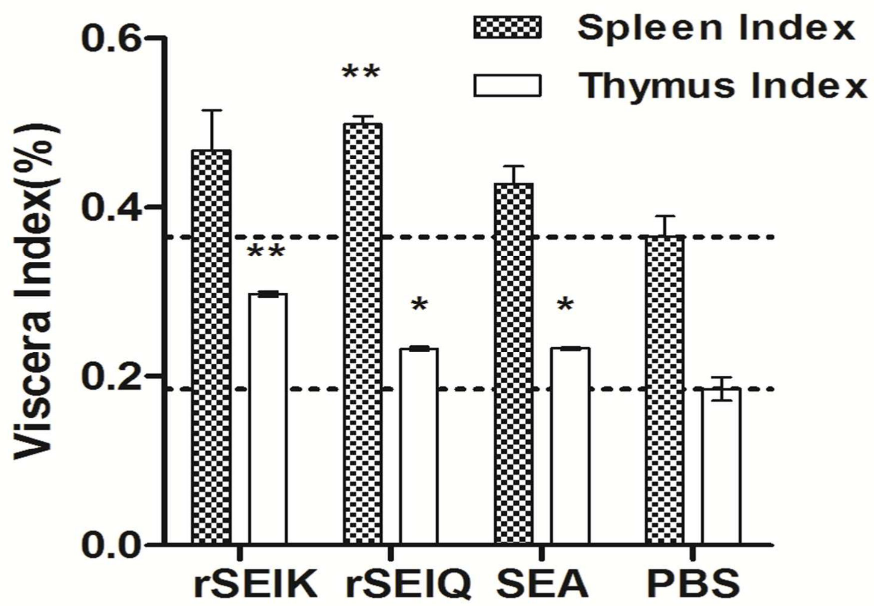

Figure 4

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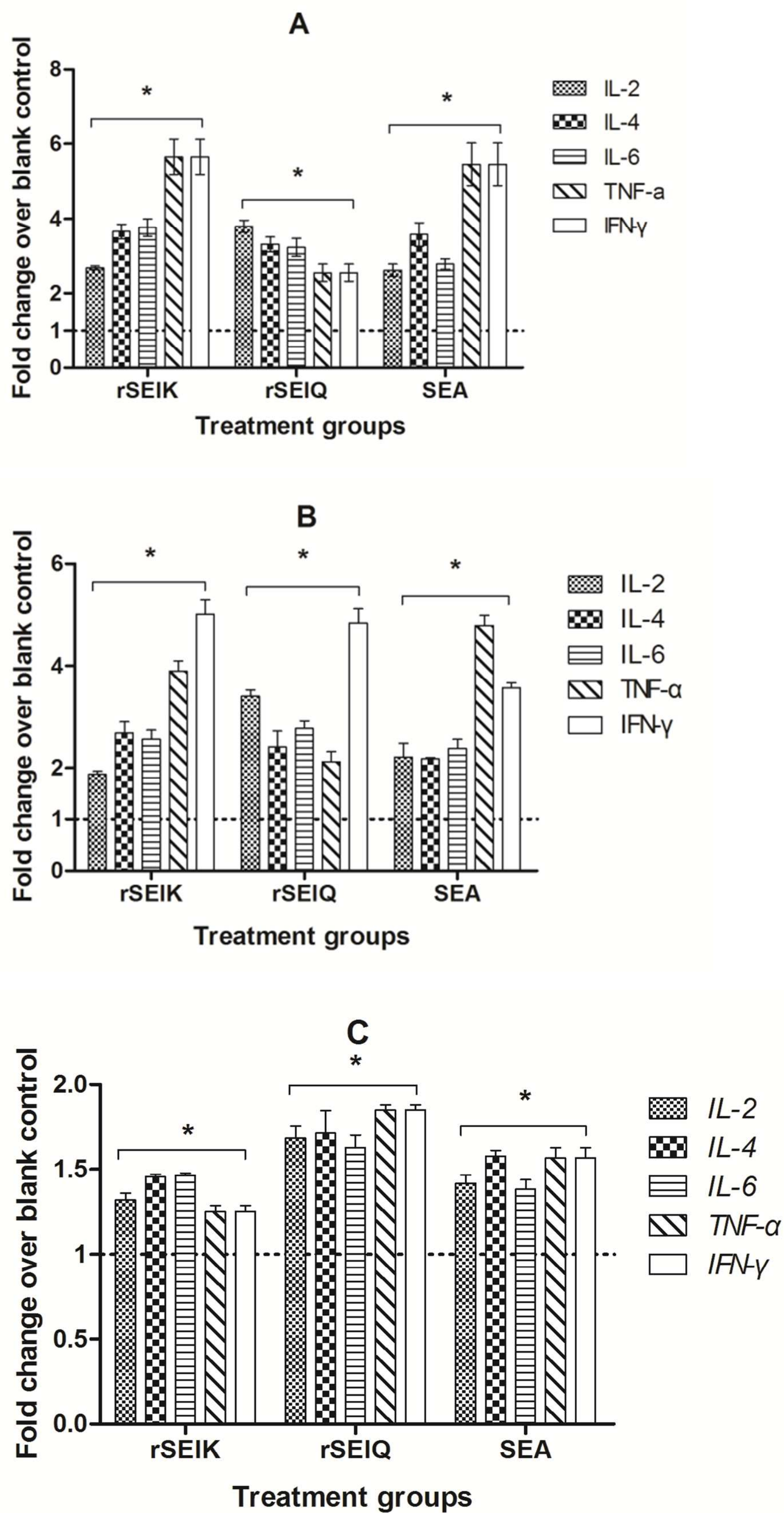

Figure 5

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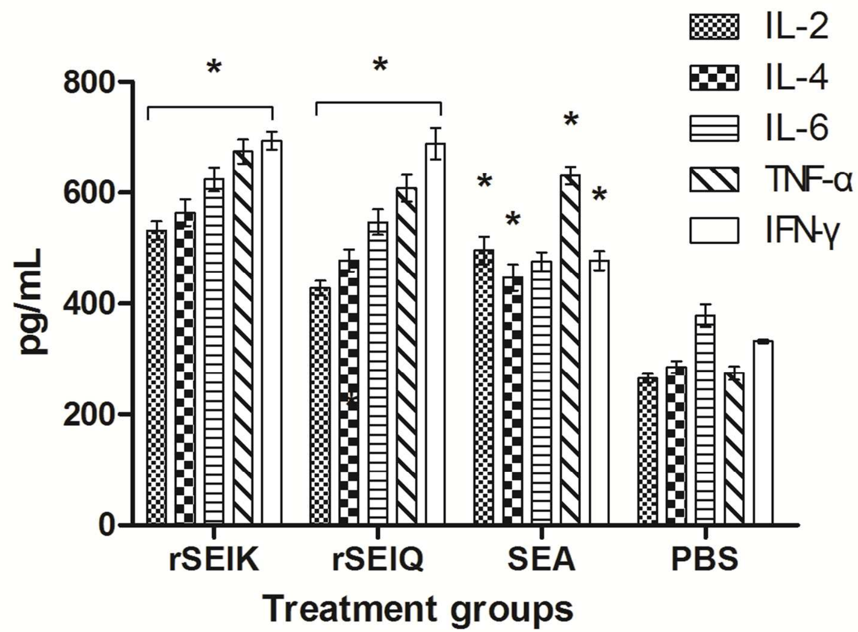

Figure 6

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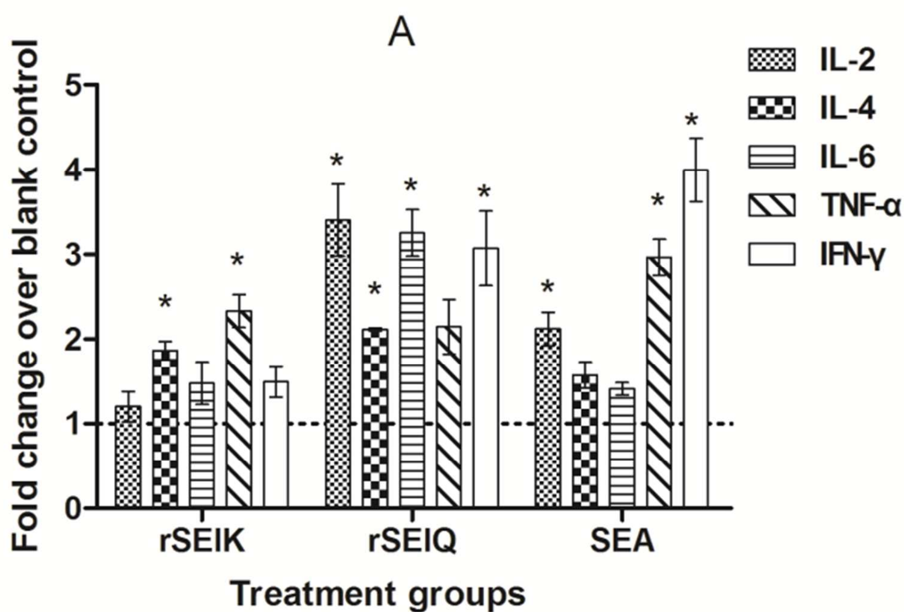

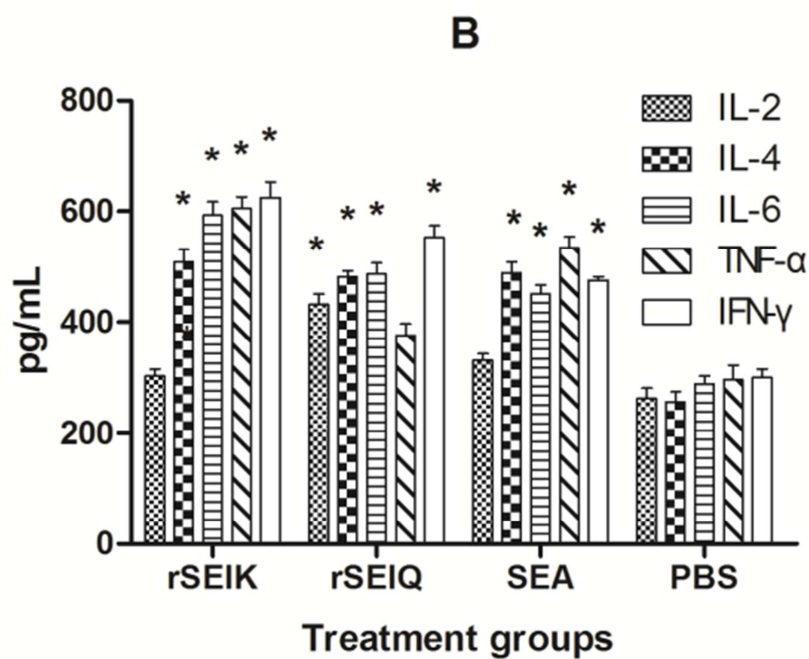

Figure 7

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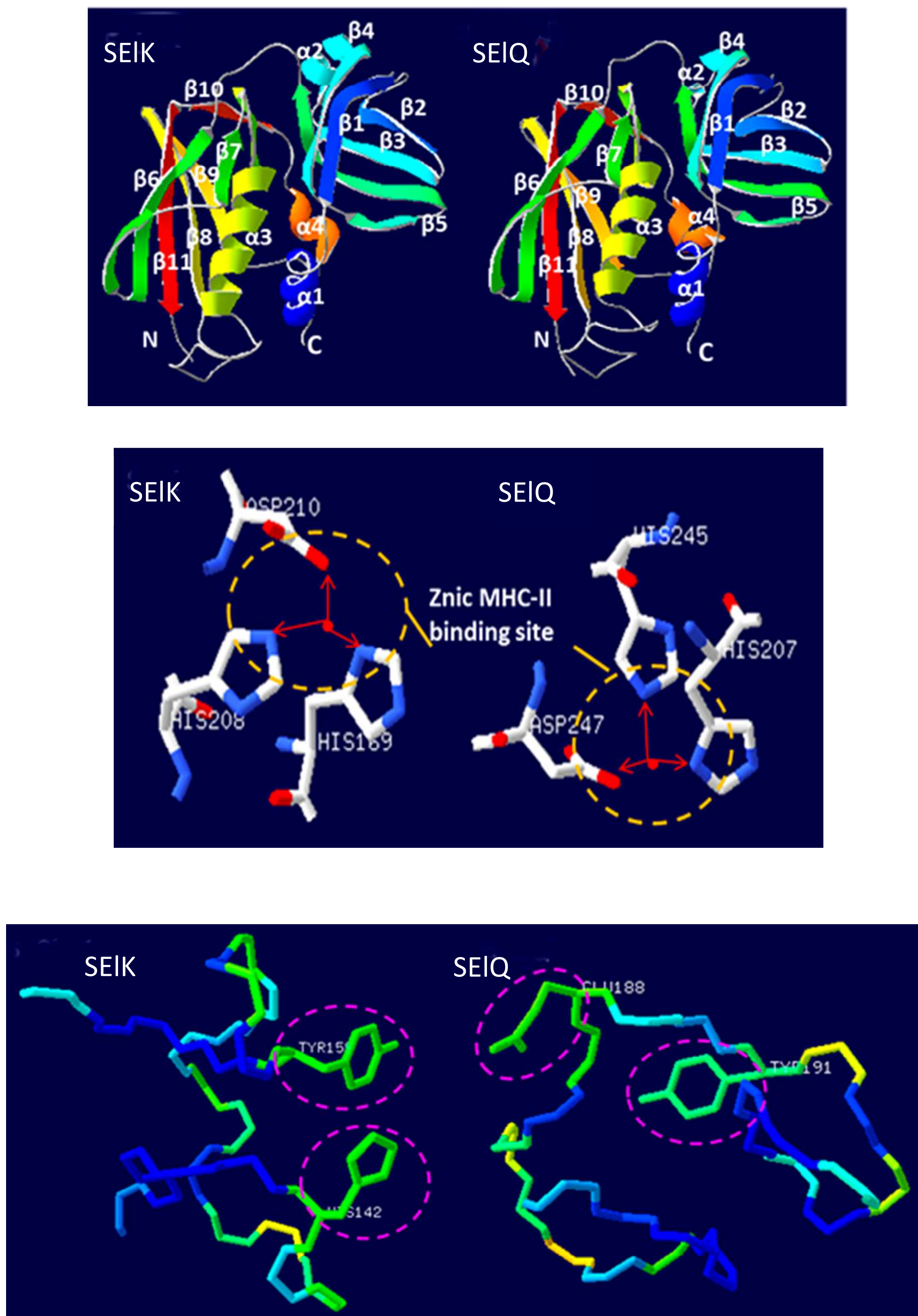

Figure 8

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Tables

Table 1 Summary of superantigenic analysis on rSElK, rSElQ treated T cells proliferation compared with inartificial SEA *in vitro*.

Group		Compositions(total volume 150 μl)
Zero adjustment		Culture solution
Tumour cell control		Culture solution, A549
Lymphocytes Background discharge	Blank	Culture solution, BalB/C or C57BL/6J thymus lymphocytes
	Positive control	Culture solution, BalB/C or C57BL/6J thymus lymphocytes, 10ng/mL ConA
	Test of SEs	Culture solution, BalB/C or C57BL/6J thymus lymphocytes, rSElK, rSElQ, SEA at concentration of 40ng/mL, A549
Lymphocytes Growth promotion with A549	Blank	Culture solution, BalB/C or C57BL/6J thymus lymphocytes, A549
	Positive control	Culture solution, BalB/C or C57BL/6J thymus lymphocytes, 10ng/mL ConA, A549
	Test of SEs	Culture solution, BalB/C or C57BL/6J thymus lymphocytes, rSElK,r SElQ, SEA at concentration of 40ng/mL, A549

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1 **Table 2 Primers of cytokines for Real-time PCR**

2

Gene	GenBank Accession No.	primer(5'-3')	Annealing Tm(°C)	Product length(bp)
IL-2	NM_008366.3	(up)GCGGCATGTTCTGGATTTGACT (down)CTCATCATCGAATTGGCACTCA	52.5	136
IL-4	M25892.1	(up)TCACAGCAACGAAGAACACCAC (down)GCATCGAAAAGCCCGAAAGAGT	54.6	155
IL-6	NM_031168.1	(up)ATGGCAATTCTGATTGTATG (down)GACTCTGGCTTTGTCTTTCT	49.8	212
IFN-γ	NM_008337.3	(up)AACTCAAGTGGCATAGATGTGGAAG (down)TGTTGACCTCAAACTTGGCAATAC	54.1	256
TNF-α	BC117057.1	(up)TGAGGTCAATCTGCCCAAGTA (down)AGGTCACTGTCCCAGCATCT	55.7	268
β-actin	NM_007393.3	(up) AGAGGGAAATCGTGCGTGAC (down)CACAGGATTCCATACCCAAG	55.9	204

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1 **Table 3 Function sites of SEs for MHC II and TCR binding.**

2

item	MHCII	TCR: α3-β8 loop	
	Znic binding key amino acids	Amino acid residues	Key amino acids
SElK	His169, His208, Asp210	26	142His,158Tyr
SElQ	His207, His245, Asp247	26	188Glu, 191Tyr

3

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