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Synergistic activation of Src, ERK and STAT pathways in PBMCs for *Staphylococcal* enterotoxin A induced production of cytokines and chemokines

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Abstract

Background: *Staphylococcal* enterotoxin A (SEA) is a well-known superantigen and stimulates human peripheral blood mononuclear cells (PBMCs) involving in the pathogenesis of inflammatory disorders and cancer.

Objective: To better understand the biological activities of SEA and the possible intracellular mechanisms by which SEA plays its roles in conditions like *staphylococcal* inflammatory and/or autoimmune disorders and immunotherapy.

Methods: Recombinant SEA (rSEA) was expressed in a prokaryotic expression system and its effects on the cytokine and chemokine production was examined by Enzyme-linked Immunospot (ELISpot) Assay and ELISA analysis.

Results: *In vitro* experiments showed rSEA could significantly enhance secretion of a broad spectrum of cytokines and chemokines from PBMCs dose-dependently. Increased secretion of cytokines and chemokines from rSEA stimulated PBMCs was barely affected by C-C motif chemokine receptor 2 (CCR2) antagonist INCB3344. However, Src, ERK and STAT pathway inhibitors were able to successfully block the enhanced secretion of most of cytokines and chemokines produced by rSEA stimulated PBMCs.

Conclusions: Our work suggested that rSEA serves as a potent stimulant of PBMCs, and induces the release of cytokines and chemokines through Src, ERK and STAT pathways upon a relatively independent network. Our work also strongly supported that Src, ERK and STAT signaling inhibitors could be effective therapeutic agents against diseases like toxic shock syndrome or infection by microbes resistant to antibiotics.

Key words: superantigen, *staphylococcal* enterotoxin A, PBMCs , cytokines and chemokines, Src, ERK and STAT pathways.

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Introduction

Staphylococcal enterotoxins (SEs) include more than 20 distinct members, such as *Staphylococcal* enterotoxin A (SEA) and *Staphylococcal* enterotoxin B (SEB). The SEA is able to activate a large population of both $CD4^+$ and $CD8^+$ T cells at very low concentrations.[1,2] It is commonly accepted that SEs bind to major histocompatibility complex (MHC) class II on antigen presenting cells and the variable region of specific Vβ regions of T-cell receptors, sequentially triggering excessive stimulation of antigen presenting cells and T-cells.[1,2] As a result, these activated immune cells produce and release massive T cell mediators, cytokines and chemokines, etc,[1-3] involving in the pathogenesis of a variety of human diseases, ranging from food poisoning,[4] inflammatory and/or autoimmune disorders,[5,6] to toxic shock,[7] and even cancer.[8] Activation of protein tyrosine kiases (PTKs) is a common step of T cell response to superantigen stimulation.

Systemic cytokine and chemokine responses induced by SEs have been demonstrated by numerous clinical and animal studies, however, whether these cytokines and chemokines can interact through a form of cross-talk, and thereafter influence the course of the diseases remained inconclusive. For example, medically relevant staphylococci, especially hospital- or community-associated methicillin resistant *Staphylococcus aureus* (MRSA), would lead to or aggravate septic shock, with a dysregulated response called "cytokine storm" or "cytokine release syndrome". Despite its pivotal role in causing sepsis, the pathophysiological role of "cytokine storm" in regulating the expression of chemokines or vice versa, is poorly understood.[9] Secondly, it has been reported that SEs stimulation of human peripheral blood monocyte cells (PBMCs) results in a rapid and dose-dependent down-regulation of chemokine receptor (i.e. CCR1, CCR2, and CCR5),[10] mediated by TNF-α,[10] in a chemokine ligand-independent mechanism.[11] It is unclear whether such a chemokine receptor down-regulation would alter the subsequent cytokine and chemokine release and activities. Lastly, there are at least three intracellular signaling pathways of T-cell activation upon superantigen binding to exert their potent effects, the phosphoinositide 3 kinase (PI3K)/mammalian target of rapamycin (mTOR), NFκB and MAPK pathways.[2,12-14] Whether the global cytokine and chemokine responses induced by SEs share the same or differential intracellular signaling pathways has remained poorly defined.

The recombinant SEs were able to provide well characterized and quantified stimulants, and significantly higher and invariable levels of cytokine and chemokine release than those challenged or vaccinated by native SEs.[4,15,16] The larger magnitude of expression of cytokines and chemokines induced by recombinant SEs should provide more insights for the toxin-induced cascades. Superantigens can induce immune cell activation and subsequent cellular changes, the same as conventional antigens. Cytokines, growth factors, chemokines released from immune cells would subsequently activate intracellular signaling pathways, resulting in inflammation and tissue damages. Our previous work found that SEA could stimulate human PBMCs to release a wide range of cytokines, and immune cell cross-talk-dependent activation of ERK and STATs in PBMCs.[3] Other researches demonstrated that Src family kinases mediate the phosphorylation of STATs and play a critical role in signal transduction pathways[16] and that Src regulates the expression of IL-1β, IL-6 and IL-8 and these effects of Src are mediated through activation of ERK-MAPK pathway.[17] Our recent work on allergen rMal f 1 proved that single antigen can simultaneously predispose DCs bias toward different pathways to inflammation.[18] IL-1β and TNF-α are relating to cell death and inflammasome activation; IL-2 is part of the body's natural response in discriminating between "self" and "non-self" via its direct effects on T cells. IL-8, CCL2 (MCP1) and MIP1A (CCL3) recruit immune cells to the sites of inflammation as response to tissue injury or infection. IL-6 and TNF-α have potential bias towards T_H22/T_H17 while IL-4 towards T_H2 and IFN-γ towards T_H1 (IP-10 is secreted by monocytes in response to IFN-γ), respectively. In this work, we therefore aimed to decipher all the possible inflammatory pathways triggered by SEA by detecting the above-mentioned cytokines and chemokines.

Methods and Materials

For detecting Human IL-1β, IL-2, IL-4, IL-6, IL-8, IFN-γ, TNF-α, CCL-2, MIP1A, and IP-10, their ELISA kits were purchased from R&D Systems (Minneapolis, Minnesota, USA). ELISpot kits for human IFN-γ and IL-4 were purchased from Mabtech AB (Sweden). Src-family kinases inhibitor PP2 and MEK/ERK inhibitor U0126 were obtained from Selleck Chemical Company (Houston,Texas, USA). Pyridone 6 (P6), a pan-JAK inhibitor, was purchased from Merck Millipore (Billerica,

Massachusetts, USA). CCR2 antagonist INCB3344 was obtained from MedChem Express (New Jersey, USA). Peripheral blood samples were from 12 healthy adult blood donors (6 female and 6 male, ranging in age from 18 to 60 years) at the Second Affiliated Hospital of Guangzhou Medical University. All donors' health condition was evaluated by the medical officials. The study protocol was reviewed and approved by the Ethical Committee of the Guangzhou Medical University and written informed consent was obtained from each participant before being included in this study.

Expression, purification and identification of recombinant SEA.

The procedure of construction, expression, and characterization of the recombinant SEA was similar to our recent report.[19] *Staphylococcus aureus* strain ATCC-13565 was used to amplify the SEA gene for cloning and expression as previously described.[20] Genomic DNA was extracted from the bacteria by using GenElute bacterial genomic DNA kit (Sigma, St Louis, USA). The mature peptide of the SEA coding region was decided according to Swiss-Prot Accession No. H6URY2 and amplified with the following pair of primers: 5'-AGCGA GAAAAGCGAAGAAAT-3' and 5'-ACTTGTATATAAATATAT AT-3'. The PCR were purified and further modified by adding the *NdeI* and *XhoI* restriction sites and Strep-tag II sequence with the following PCR primers: F2 5'- GGAATTCCATATG AGCGAGAAAAGCGAAGAAAT -3' (with *NdeI* site underlined); R2, 5'- CCGCTCGAGTTATTATTTTTCGAACTGCGG GTGGCTCCAACTTGTATATAAATATATAT -3' (with *XhoI* site underlined and Strep-tag II sequence in bold italic). The modified products were inserted into the pET44a protein expression vector. The rSEA was expressed in the *Escherichia coli* strain Rosetta-gami 2 and purified by using Strep-Tactin affinity chromatography and its sequences were identified by liquid chromatography with mass spectrometry analysis (LC-MS/MS Analysis). To remove any contaminating LPS, rSEA protein was purified by High-Capacity Endotoxin Removal Resin (Thermo Fisher Scientific). The LPS content was analyzed by the TachypleusAmebocyte Lysate (TAL) method. Samples that contained less than 0.5 EU/ mg LPS were deemed to be suitable for use in the following assays.

Cytokines Analysis by Enzyme-linked Immunospot (ELISpot) Assay

The procedure was carried out similar to our previous work.[3] Briefly, PBMCs were separated from heparinized blood obtained from healthy donors by Ficoll-Paque PLUS (GE Healthcare, USA) density gradient centrifugation. Generally in this study PBMC were seeded in triplicates into wells for each test and each experiment was repeated three times.

Human IFN-γ and IL-4 secreting cell numbers were determined using ELISpot kits. Briefly, 96-well nitrocellulose -bottomed plates were coated with murine anti-human IFN-γ (1-D1K) or IL-4 monoclonal antibody (IL-4I) at a concentration of 15 μg/mL in PBS and incubated at 4°C. After 24 h, the plates were washed 5 times with sterile PBS and blocked with RPMI containing 10% fetal bovine serum (FBS). Then PBMCs were distributed into 96-well nitrocellulose-bottomed plates (3×10^5 cells/well) with addition of rSEA at different concentrations from 1 pg/mL to1 μg/mL and we used anti-CD3mAb (100ng/mL) as a positive control.

After an 18 h incubation and being washed five times with PBST (10 mmol/L PBS pH7.4, 0.1% Tween-20), the plates were incubated with biotinylated anti-human IFN-γ (7-B6-1) or IL-4 mAb (IL-4II) at a concentration of 1 μg/mL at room temperature for 1h, followed by streptavidin-horseradish peroxidase (1:1000 in dilution buffer) incubation at room temperature for 1h and washed as previously. Finally, the color was developed by TMB solution, after the spots were developed for 10 to 15 min, the plates were washed with distilled water and air-dried. The number of antigen-specific IFN-γ-secreting (or IL-4-secreting) spot forming cells was determined with a computer-assisted video imaging ELISpot reader (AID, German).

Cytokines Analysis by ELISA

Freshly isolated PBMCs in 24-well plates (5×10^5 cells/well) were stimulated by rSEA at 100 ng/mL for 15 min and 1, 3, 6 and 24 h. Then cell culture supernatants were harvested and cytokine levels were measured by ELISA. IL-1β, IL-2, IL-4, IL-6, IL-8, IFN-γ, TNF-α, CCL-2, MIP1A and IP-10 concentrations in cell culture supernatants were measured using ELISA assay following the manufacturer's instructions. For experiments with CCR2 antagonist INCB3344, Src-family kinases inhibitor PP2, MEK/ERK inhibitor U0126 or pan-JAK inhibitor P6 treatment, PBMCs were pre-incubated with INCB3344 (10 nM, 100 nM, 1 μM, 10μM), PP2 (0.4, 2, 10μM), U0126 (0.4, 2, 10, 20 μM) or P6 (5 nM, 50 nM, 500 nM, 5 μM) for 2 h and then stimulated with 100 ng/mL rSEA for 24 h still in the presence of INCB3344, PP2, U0126 or P6.

Statistical Analysis

GraphPad Prism Software was used for statistical comparisons with one-way ANOVA followed by *post hoc* analysis. All quantitative data was presented as mean ± SEM, which were from three independent experiments. Statistical significance was concluded if P value < 0.05.

Results

Production of recombinant SEA

In the present study, to obtain a large quantity and high purity of materials, the size of 31 kDa SEA protein was produced in the *Escherichia coli* and purified by using Strep-Tactin affinity chromatography (**Figure 1A**). After purification by High-Capacity Endotoxin Removal Resin, the concentration of LPS was 0.061 EU/ mg as detected by the TachypleusAmebocyte Lysate (TAL) method. To identify the peptide sequences, rSEA was processed with tryptic digestion and subsequent LC-MS/MS analysis. The Full-scan mass spectrum of a representative peptide fragment in the tryptic digests derived from rSEA (T3) was shown in **Figure 1B**.

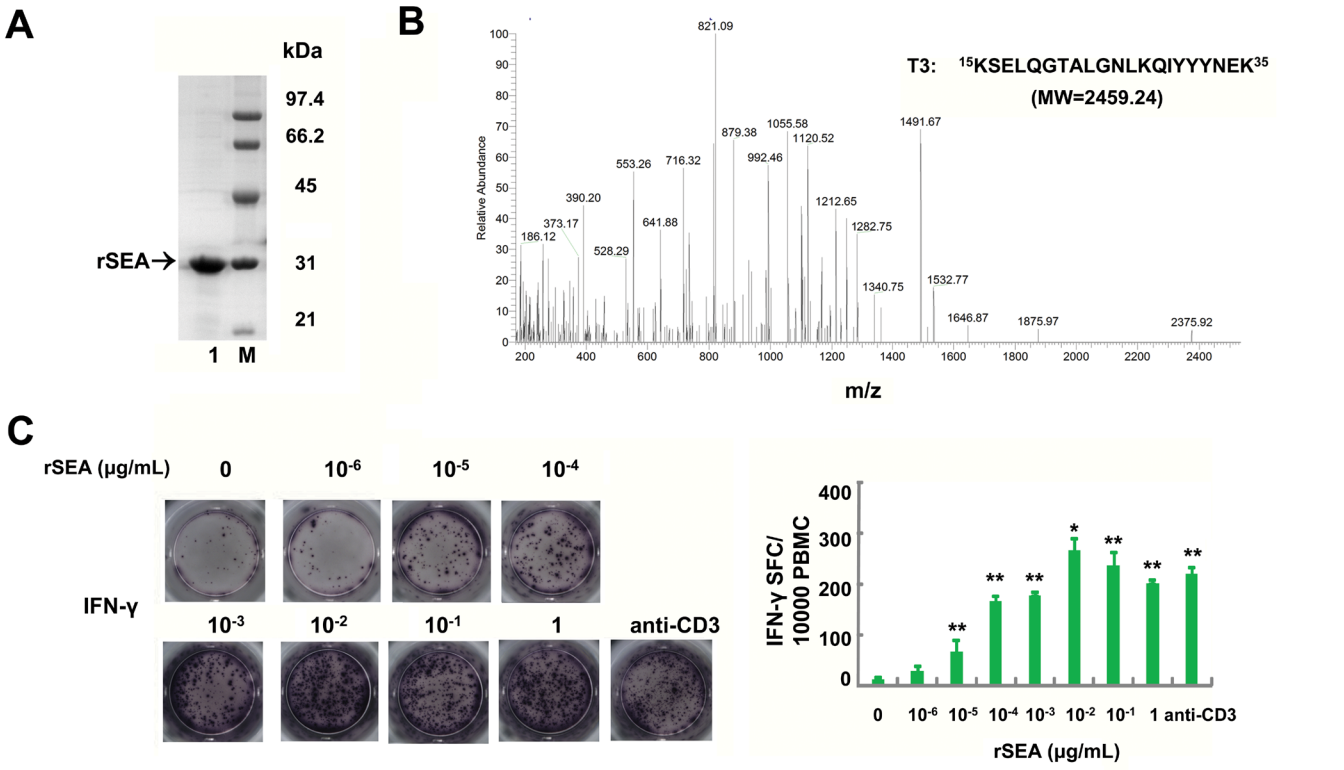

Figure 1. ***In vitro*** **activation of human PBMCs stimulated by different concentrations of rSEA.** ***A***, SDS-PAGE analysis of purified rSEA. ***B***, Full-scan ion evaporation mass spectra of T3 peptide fragments derived from the tryptic digests of rSEA.

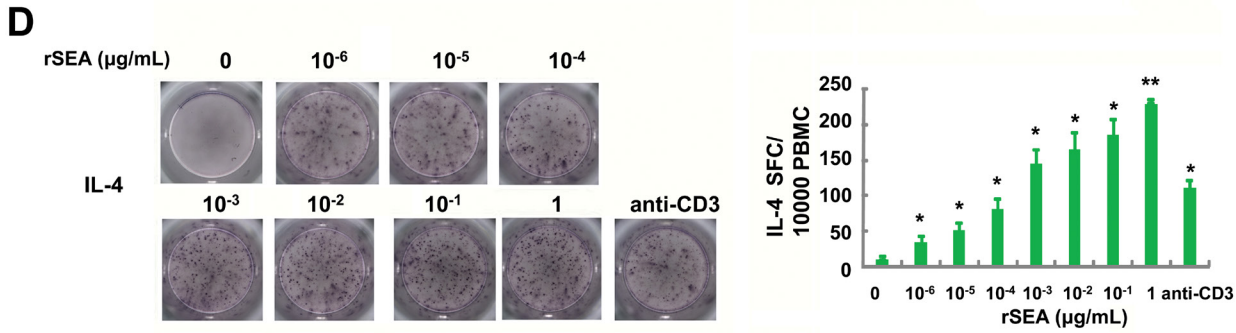

Figure 1. (Continued) ***C-D***, Measurement of IFN-γ (***C***) and IL-4 (***D***) secretion by ELISpot assay. Representative images of ELISpot wells were shown on the left and histograms of IFN-γ and IL-4 secreting spot forming cells (SFC) per 10^4 PBMC were shown on the right. rSEA were used at concentrations from 1 pg/mL to 1 μg/mL. In C-D, * $P < 0.05$, ** $P < 0.01$ compared to PBMCs stimulated with PBS (one-way ANOVA followed by *post hoc* analysis, referred as "ANOVA" in following figure legends).

SEA increases IFN-γ and IL-4-secreting cell numbers in human PBMCs by different concentrations

ELISpot assay is a widely used method for quantification of cytokine-secreting cell numbers and monitoring cellular immune responses.[21] In the present study, human PBMC (3×10^5 cells/well) were incubated for 18 h in the absence (served as controls) or presence of rSEA (at concentrations from 1 pg/mL to 1 μg/mL) and the number of spot forming cells (SFC) secreting IFN-γ (**Figure 1C**) and IL-4 (**Figure 1D**) were analyzed by ELISpot assays. The results demonstrated that significantly higher numbers of IFN-γ (**Figure 1C**) and IL-4 (**Figure 1D**) spots were detected in PBMCs incubated with rSEA than that in controls. Also the number of spots secreting IFN-γ and IL-4 were seen in a dose-dependent manner stimulated by rSEA with PBMCs (**Figure 1C-D**).

SEA induces the secretion of cytokines and chemokines in human PBMCs at different time points

As the ELISPOT data shown in **Figure 1C-D**, 100 ng/mL of rSEA was the intermediate concentration to induce cytokines release by PBMCs. This concentration was decided as the dose for further rSEA and PBMCs co-culture assay and time-course experiments to investigate more kinds of cytokines and chemokines secreted from PBMCs. Briefly, PBMCs (5×10^5 cells/well) were stimulated with 100 ng/mL rSEA for 15 min and 1, 3, 6 and 24 h. A more automated assay,[21] ELISA was used to measure the levels of cytokines IL-1β, IL-2, IL-4, IL-6, IFN-γ, TNF-α and chemokines CCL-2, IL-8, MIP1A, IP-10 in cell culture supernatants. The results showed all cytokines and chemokines detected increased gradually over time, and peaked at 24 h. (**Figure 2A, B**).

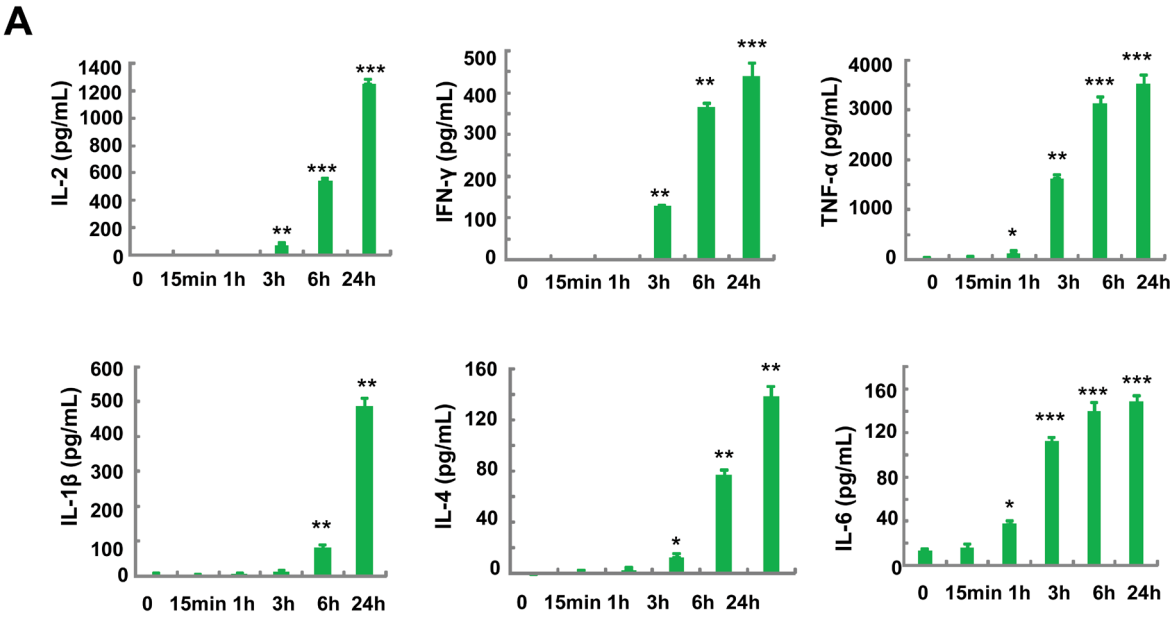

Figure 2. rSEA induces the secretion of cytokines and chemokines in human PBMCs at different time points. ***A***, rSEA induced secretion of cytokines (IL-2, IFN-γ, TNF-α, IL-1β, IL-4 and IL-6) in human PBMCs.

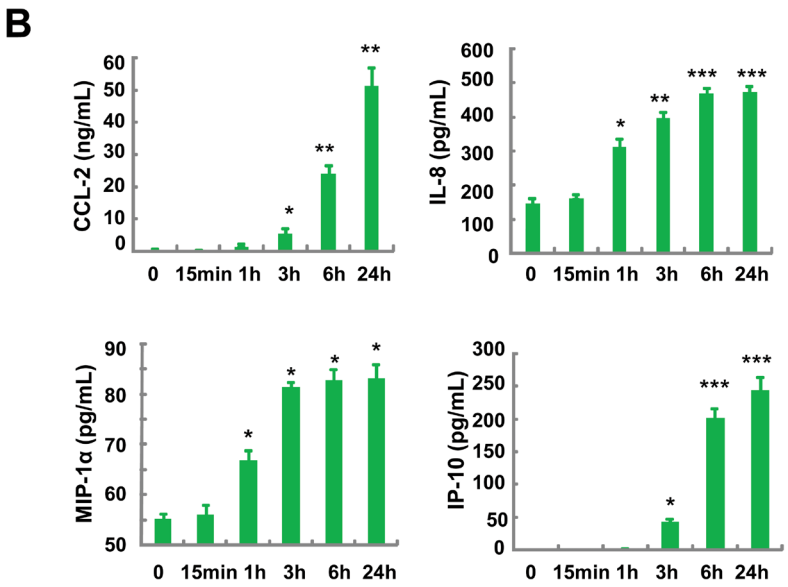

Figure 2. (Continued) ***B***, rSEA induced the secretion of chemokines (CCL-2, IL-8, MIP1A, IP-10) in human PBMCs. * P < 0.05, ** P < 0.01 , ***P < 0.001 compared with PBMCs stimulated without rSEA (ANOVA).

Effect of CCR2 antagonist INCB3344 on rSEA-induced cytokine and chemokine production

A previous study reported INCB3344 as a novel, potent and selective CCR2 antagonist; it inhibited the binding of CCL2 to mouse PBMCs in a dose-dependent manner.[22] Here we also used INCB3344 to examine whether blocking the CCR2 would significantly influence the production of rSEA-induced cytokines and chemokines. The results of the ELISA assays indicated the CCR2 antagonist INCB3344 was barely able to significantly change the production of all cytokines and chemokines (**Figure 3A, B**). The only exception is at the highest dose (10 μM), INCB3344 can inhibit secretion of some rSEA-induced cytokines and chemokines, including IL-2, TNF-α, IL-4,IL-6 and IL-8 slightly (**Figure 3A and 3B**).

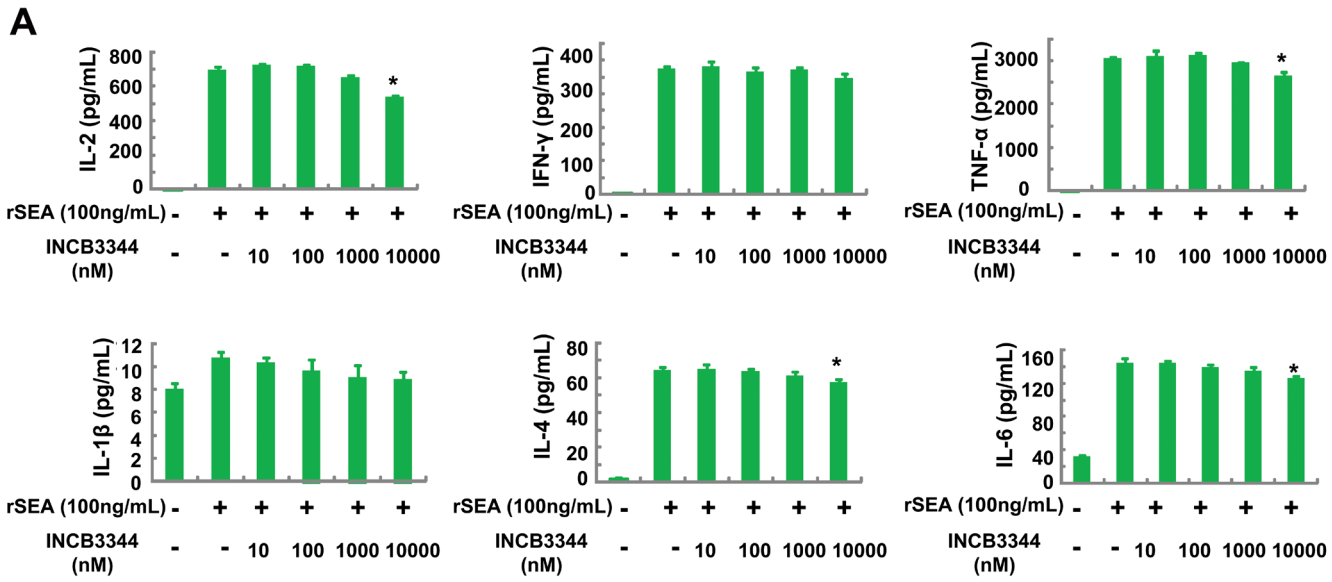

Figure 3. Effect of CCR2 antagonist INCB3344 on the productionof rSEA-induced cytokines and chemokines. PBMCs were pre-incubated with INCB3344 (10 nM, 100 nM, 1 μM and 10μM) for 2 h and then stimulated with 100 ng/mL rSEA for 24 h still in the presence of INCB3344. Determination the concentrations of detected cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ, TNF-α) (***A***) and chemokines (CCL-2, IL-8, MIP1A, IP-10)

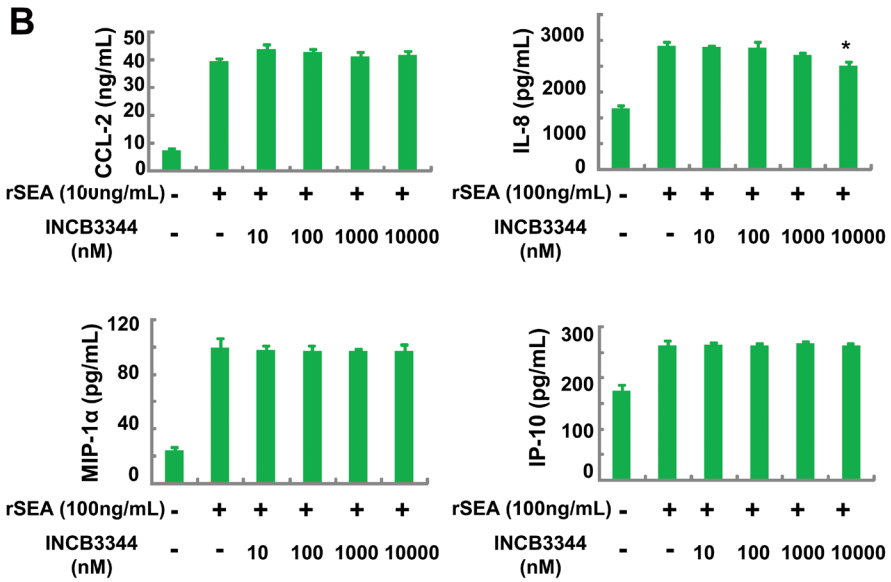

Figure 3. (Continued) (***B***) in cell culture supernatants by ELISA. * $P < 0.05$ compared with PBMCs stimulated with 100 ng/mL rSEA without INCB3344 (ANOVA).

Effect of Src-family kinases inhibitor PP2 on the production of rSEA-induced cytokines and chemokines

Next, we used antagonists or inhibitors of intracellular signaling pathways to further characterize the pathway of rSEA-induced cytokine release. A Src-family kinases inhibitor (PP2)[23] was used and the results of ELISA assays indicated that Src-family kinases inhibitor PP2 (2-10 μM) could inhibit the secretion of all detected rSEA-induced cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ, TNF-α) and chemokines (CCL-2, IL-8, MIP1A, IP-10), significantlyat the concentrations of 10 μM of PP2 (**Figure 4A and 4B**).

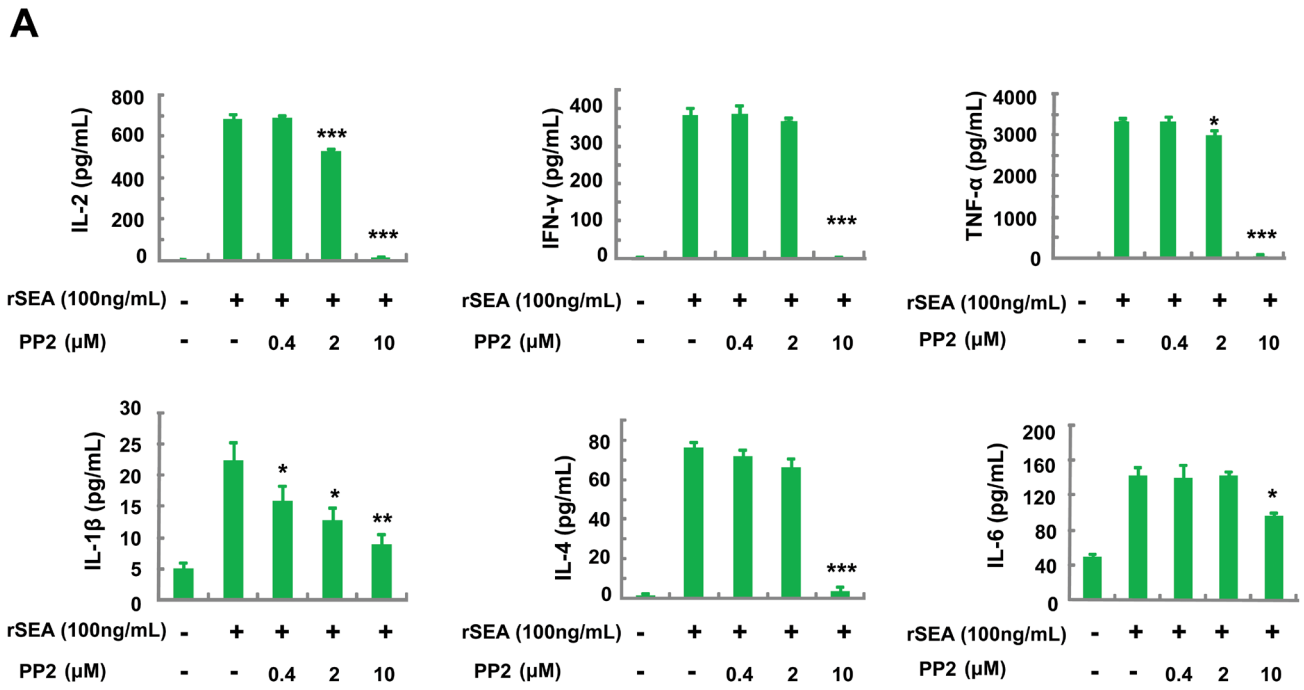

Figure 4. Effect of Src-family kinases inhibitor PP2 on rSEA-induced cytokine and chemokine production. PBMCs were pre-incubated with PP2 (0.4, 2, 10 μM) for 2 h and then stimulated with 100 ng/mL rSEA for 24 h still in the presence of PP2. Determination the concentrations of detected cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ, TNF-α) (***A***) and chemokines (CCL-2, IL-8, MIP1A, IP-10)

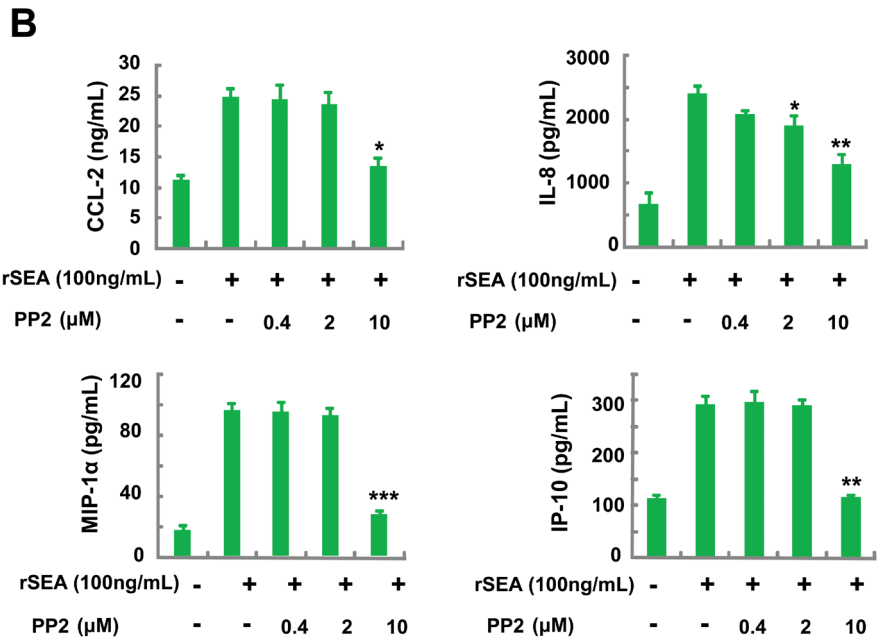

Figure 4. (Continued) (*B*) in cell culture supernatants by ELISA. * P< 0.05, ** P<0.01, ***P< 0.001 compared with PBMCs stimulated with 100 ng/mL SEA without PP2 (ANOVA).

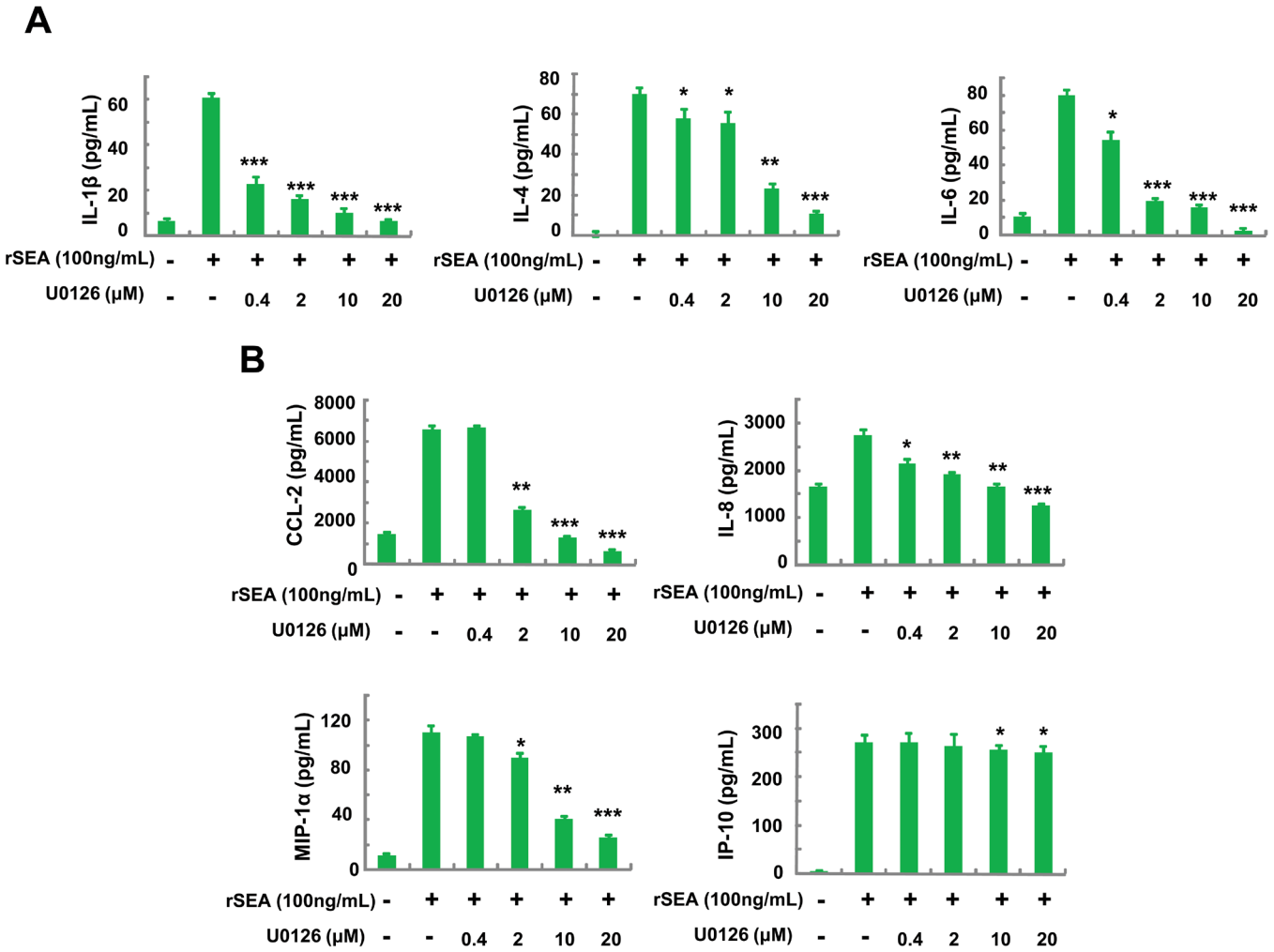

Figure 5. Effect of MEK inhibitor U0126 on rSEA-induced cytokine and chemokine production. PBMCs were pre-incubated with U0126 (0.4, 2, 10, 20 μM) for 2 h and then stimulated with 100 ng/mL rSEA for 24 h still in the presence of U0126. Determination the concentrations of detected cytokines (IL-1β, IL-4, IL-6) (*A*) and chemokines (CCL-2, IL-8, MIP1A, IP-10) (*B*) in cell culture supernatants by ELISA. * P< 0.05, ** P<0.01, ***P< 0.001 compared with PBMCs stimulated with 100 ng/mL rSEA without U0126 (ANOVA).

Effect of MEK inhibitor U0126 on rSEA-induced cytokine and chemokine production

Our previous work reported rSEA acted through ERK and STAT pathways to exert anti-tumor activity.[3] Similarly, we used the MEK inhibitor U0126 and the results of the ELISA assays indicated that U0126 (0.4-20 μM) could inhibit rSEA-induced secretion of cytokines (IL-1β, IL-4, IL-6) and chemokines (CCL-2, IL-8, MIP1A, IP-10) in a dose-dependent manner (**Figure 5**). Notably, our previous published data showed that when PBMCs were treated with rSEA in the presence of 20 μM U0126, the production of TNF-α, IFN-γ and IL-2 was completely abolished by the MEK inhibitor.[3]

Effect of pan-JAK inhibitor P6 on rSEA-induced cytokine and chemokine production

We also applied pan-JAK inhibitor P6 to check its possible role. Our data showed that P6 significantly reduced rSEA-induced cytokine and chemokine secretion in a dose dependent manner detected by ELISA assays (**Figure 6**). Again, our previously published data showed that P6 significantly reduced rSEA-induced TNF-α and IFN-γ secretion dose dependently as detected by ELISA assays, but it inhibited IL-2 secretion only at the highest dose.[3]

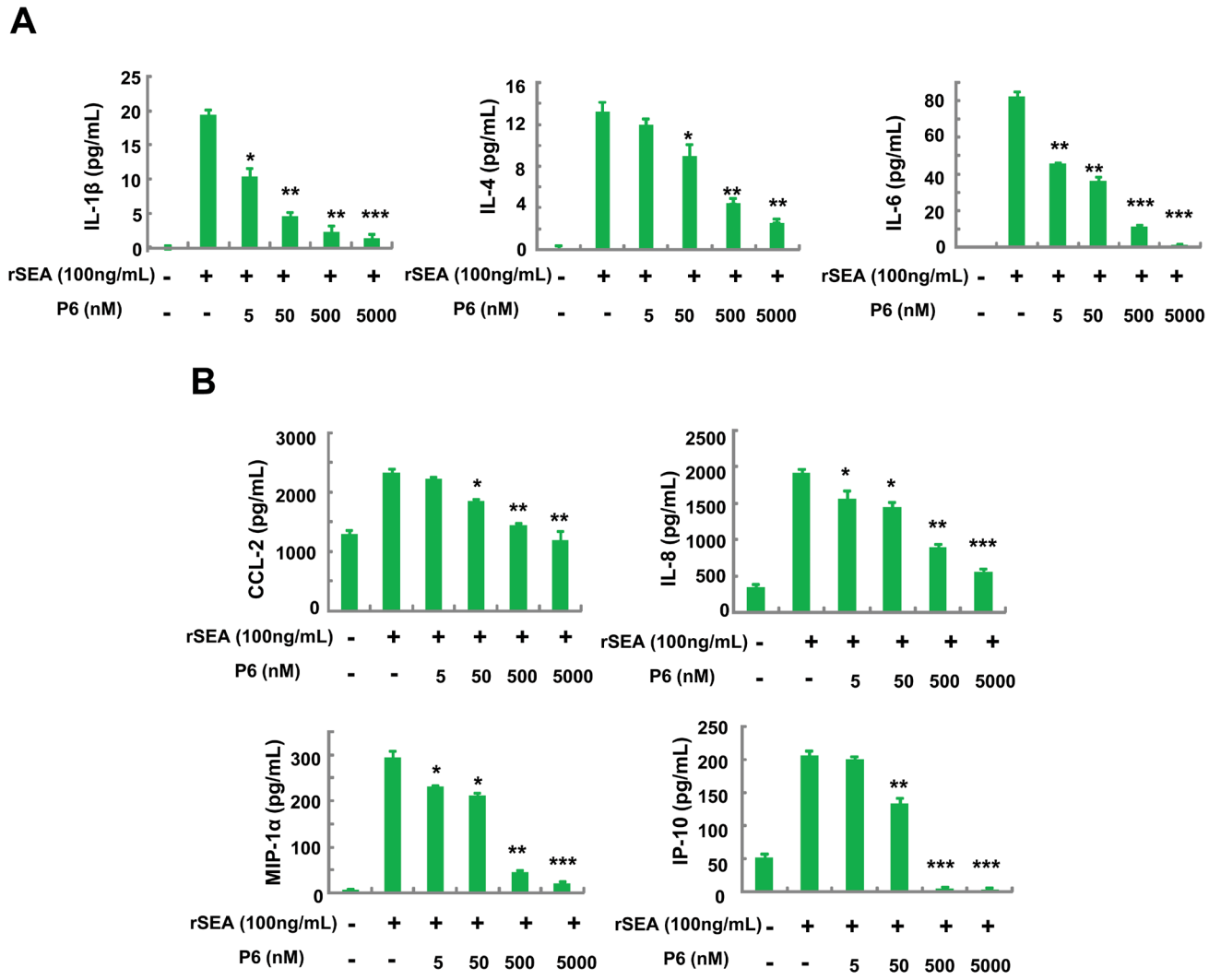

Figure 6 Effect of pan-JAK inhibitor P6 on rSEA-induced cytokine and chemokine production. PBMCs were pre-incubated with P6 (5 nM, 50 nM, 500 nM, 5μM) for 2 h and then stimulated with 100 ng/mL rSEA for 24 h still in the presence of P6. Determination the concentrations of detected cytokines (IL-1β, IL-4, IL-6) (***A***) and chemokines (CCL-2, IL-8, MIP1A, IP-10) (***B***) in cell culture supernatants by ELISA. * P< 0.05, ** P<0.01, ***P< 0.001 compared with PBMCs stimulated with 100 ng/mL rSEA without P6 (ANOVA).

Discussion

Staphylococcal enterotoxin A (SEA), as a biomarker of *Staphylococcus aureus*, is among the most potent T cell activators ever known.[1,2] Recently, SEA protein fused to Fab fragments of monoclonal antibodies, or recombinant SEA by engineering technology, have been applied to *in vitro* or animal studies. Such attempts helped us better understand the role of SEA in the pathogenesis of multiple clinical syndromes ranging from acute food poisoning,[4] atopic dermatitis,[5] to highly lethal toxic shock,[7] and even cancer.[8] To obtain SEA in a fast, accurate and low cost way, we produced recombinant SEA (rSEA) by cloning the coding sequence into a pET44a vector, and transforming the coding sequence into *E. coli* strain Rosetta-gami 2. The SEA sequences were confirmed by the LC-MS/MS analysis. Such purified and identified rSEA could provide an efficient tool for us to study research projects in immunotherapy and atopic dermatitis, etc.

Increasing evidence has shown that T cells release excessive cytokines and chemokines in response to bacterial exotoxins, resulting in conditions like streptococcal toxic shock syndrome.[7] SEA and SEB are particularly capable of inducing cytokine/chemokine release from T-cells.[7,24-27] For example, SEB could robustly activate $CD4^+$ and $CD8^+$ T cells contributing to the cytokine storm and toxic shock.[7,27] Previously Rajagopalan et al reported that 28 of 30 cytokines in total or 27 of 30 chemokines in total as examined were significantly elevated at 3 h after SEB challenge in mice.[27] Consistent with previous work,[8,16,18] the present study also indicated that SEA was able to induced a prompt, non-specific and profound activation of PBMCs and secretion of cytokines and chemokines from PBMCs, and the latter occurred in a dose-dependent manner and lasted a long term (> 24 h *in vitro*). Moreover, it was revealed by previous studies that at least several proinflammatory factors, i.e., IFN-γ,[25] TNF-α,[27] and IL-8[26] played a leading role in the production of other cytokines and chemokines. Collectively, these studies supported the notion that the cytokines induced by superantigen would further potentiate the production of chemokines.

Chemokines, master regulators in a number of microenvironment responses, are small polypeptides produced by various cell types. Previous studies reported that superantigens caused a rapid, dose-dependent down-regulation of chemokine receptor (i.e., CCR1, CCR2, and CCR5) binding sites while stimulating PBMCs.[10] INCB3344, a novel, potent and selective CCR2 antagonist, inhibited the binding of CCL2 to mouse PBMCs in a dose-dependent manner.[22] Our results indicated that, even though blocking a certain chemokine receptor (i.e., CCR2), the rSEA induced systemic cytokine and chemokine responses were largely unaffected. Therefore, it is less likely that one or two chemokines production or their receptors down-regulation would alter other cytokine and chemokine production and activities induced by superantigens. In all, our work strongly support the notion that a cognate interaction of cytokine and chemokine network still presents and represents one mechanism of communication between PBMCs and superantigens stimulation.

There are at least three intracellular signaling mediators for T-cell activation upon superantigens binding; the phosphoinositide 3 kinase (PI3K)/mammalian target of rapamycin (mTOR), NFκB and MAPK pathways.[2,12-14] The cytokines and chemokines produced by superantigen stimulated PBMCs act through their individual receptors and distinct receptor mediated signaling pathways. However, there was considerable evidence to support the involvement of cross-talk between multiple signaling pathways. For example, the MAPK family includes JNK, ERK, and p38. Inhibiting p38 and ERK pathway almost completely blocked the effect of SEB in regulating the production of proinflammatory cytokines.[28] Exposure to *Staphylococcal* enterotoxins-PBMC-conditioned medium resulted in STAT and the Janus protein-tyrosine kinase (Jak) activation.[29,30] Fyn, a member of Src family, in the T-cells also activated by SEs for the signaling process,[31] and SEA induced activation of STAT3 in human $CD4^+$ T cell lines could be almost completely inhibited by a Src kinase inhibitor (PP1).[23] Our previous work reported rSEA could exert its potent anti-tumor activity via the cooperation of ERK and STAT pathways.[3] To further elucidate the cellular mechanisms contributing to T-cells activation and sequential immune response, in this study, we applied a few antagonists or inhibitors to the signaling pathways and their effects on the production of the cytokines and chemokines was examined. Our data showed that rSEA-induced release of cytokines and chemokines from PBMCs could be dose-dependently inhibited by any of these three intracellular signaling pathways. Combined with previous findings,[3,4,8,32] our data support the hypothesis that Src, ERK and STAT pathways convey signaling responses in an independent but also synergistic manner. There were only a few similar studies checking network cross-talk among triple or even more pathways in a study,[15,33] however, to our knowledge there is no such literature ever used SEA or SEB. Thus, our work is the first study attempting to link the network of Src, ERK and STAT pathways together for SEA or SEB stimulation of the immune responding cells.

Other than toxic shock syndrome, many refractory diseases, such as Guillain-Barré syndrome, myasthenia gravis and corticosteroid-resistant dermatomyositis, etc. are all associated with hyperinflammatory states.[2,12,34] Antibiotic treatment has been complicated by the development of resistant strain (such as methicillin-resistant *S. aureus* (MRSA)) infection, which represents one of the most costly diseases in USA.[2,12,34] There are numerous experimental approaches attempting to block cytokine storm including trying to interfere with the earliest events, or more directly, to reduce the synthesis of cytokines. One "old" approach is the use of low-dose steroids. For example, a FDA-approved immunosuppressive corticosteriod, dexamethasone, has been used clinically for various inflammatory diseases.[12,35] Dexamethasone potently inhibited SEA- and SEB-induced cytokine release and T-cell proliferation in human PBMCs.[12,35] Another important way is to use inhibitors to block signal transduction pathways activated by super antigens.[2,12,34] The obvious advantage is that many different superantigens or even pathogens eliciting similar host responses or pathways could be effectively inhibited. Our work indicated, therapeutic inhibitors of Src, ERK and STAT signal transduction might be potential therapeutic approaches against inflammatory diseases and carcinoma, especially to treat those cases with microbial resistance against antibiotics or to spare steroid usage. Follow-up studies, for instance, using an animal model,

to see if those inhibitors have any effect against any inflammatory diseases, or against any type of cancer, would be interesting to work with.

In conclusion, we obtained a recombinant SEA (rSEA) in a fast, accurate and low cost way and confirmed this purified rSEA could significantly promote proliferation of PBMCs and enhance secretion of different cytokines and chemokines. Our studies indicated blocking CCR2 cannot alter other cytokine and chemokine production from PMBCs induced by rSEA *in vitro*. However, Src, ERK and STAT pathway inhibitors all successfully blocked the enhanced secretion of most of cytokines and chemokines produced by rSEA stimulated PBMCs.

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Declaration of conflicting interests

The authors report no conflict of interest.

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