Structural basis for the neutralization and specificity of Staphylococcal enterotoxin B against its MHC Class II binding site

Tian Xia1,2,†, Shuaiyi Liang3,†, Huajing Wang4,6, Shi Hu4,6, Yuna Sun5, Xiaojie Yu4,6, Jun Han2, Jun Li2, Shangjing Guo2, Jianxin Dai1,2,4,*, Zhiyong Lou3,*, and Yajun Guo1,2,4,*

1International Joint Cancer Institute; Second Military Medical University; Shanghai, P.R. China; 2 College of Pharmacy; Liaocheng University; Liaocheng, P.R. China; 3Laboratory of Structural Biology and MOE Laboratory of Protein Science; School of Medicine and Life Science; Tsinghua University; Beijing, P.R. China; 4State Key Laboratory of Antibody Medicine and Targeting Therapy and Shanghai Key Laboratory of Cell Engineering and Antibody; Shanghai, P.R. China; 5National Laboratory of Macromolecules; Institute of Biophysics; Chinese Academy of Science; Beijing, P.R. China

†These authors contributed equally to this work.

Keywords: Staphylococcus aureus; enterotoxin B; neutralizing antibody; crystal structure; mechanism

Introduction

Staphylococcal enterotoxin (Se) B is among the potent toxins produced by Staphylococcus aureus that cause toxic shock syndrome (TSS), which can result in multi-organ failure and death. Currently, neutralizing antibodies have been shown to be effective immunotherapeutic agents against this toxin, but the structural basis of the neutralizing mechanism is still unknown. In this study, we generated a neutralizing monoclonal antibody, 3E2, against SeB, and analyzed the crystal structure of the SeB-3E2 Fab complex. Crystallographic analysis suggested that the neutralizing epitope overlapped with the MHC II molecule binding site on SeB, and thus 3E2 could inhibit SeB function by preventing interaction with the MHC II molecule. Mutagenesis studies were done on SeB, as well as the related Staphylococcus aureus toxins SeA and SeC. These studies revealed that tyrosine (Y)46 and lysine (K)71 residues of SeB are essential to specific antibody–antigen recognition and neutralization. Substitution of Y at SeA glutamine (Q)49, which corresponds to SeB Y46, increased both 3E2’s binding to SeA in vitro and the neutralization of SeA in vivo. These results suggested that SeB Y46 is responsible for distinguishing SeB from SeA. These findings may be helpful for the development of antibody-based therapy for SeB-induced TSS.
for the prevention of host cell intoxication. Human intravenous immunoglobulin (Ig) is sometimes effective in the treatment of SAo-related inflammatory diseases. Moreover, specific antibodies given passively to naive mice or non-human primates concomitant to or hours after toxin exposure have been shown to protect against toxic shock. SEB-neutralizing monoclonal antibodies (mAbs) typically act by sterically blocking protein–protein interactions and therefore require a high affinity to compete with the TCR and MHC class II affinity reactions of host cell activation. Several well-characterized neutralizing antibodies, such as 20B10, 82M.1.2, and MAb5, act by binding to epitopes that are identical or overlap with the surfaces that recognize the cellular receptor. Their exact neutralizing mechanisms, however, remain unknown. Understanding the mechanisms by which antibodies specifically neutralize SEB requires detailed knowledge not only of the SEB structure, but also of the SEB–antibody interactions at the amino acid level.

To gain a better understanding of these mechanisms, we performed in vitro and in vivo studies of 3E2, a neutralizing mAb that recognizes SEB with high specificity and sub-nanomolar affinity. We found that 3E2 strongly inhibits SEB-induced T cell proliferation and activation. We subsequently determined the crystal structure of the 3E2 Fab in complex with SEB, and obtained detailed molecular insights into the inhibition and specific recognition mechanisms. The structural information provided in this study may help in the development of antibody-based therapies for SEB-induced TSS.

### Results

#### Identification of 3E2 as a neutralizing antibody against SEB

Recombinant SEB proteins were expressed in soluble form in prokaryotic cells and purified for preparation with a toxoid immunogen to generate mAbs capable of neutralizing SEB. Ten hybridomas were obtained and screened for the production of anti-SEB mAbs using enzyme-linked immunosorbent assay (ELISA) with purified SEB (Fig. 1A). In addition, serial dilutions of mAbs were assessed for their ability to inhibit SEB-induced T cell mitogenesis in human peripheral blood mononuclear cells (PBMCs) (Fig. 1B). Six mAbs, 3C1, 1D1, 2H4, 3E2, 4A3, and 1A5, were initially found to inhibit SEB-induced mitogenesis in a dose-dependent manner. 3E2 showed the greatest inhibitory effect among these mAbs. Primary human
T cells stimulated with SEB in vitro upregulated the secretion of cytokines, including interferon (IFN)-γ and tumor necrosis factor (TNF). Seven mAbs, 3C1, 1D1, 2G5, 2H4, 3E2, 4A3, and 1A5, showed significant inhibitory effect on SEB-induced cytokine production ($P < 0.01$), whereas an irrelevant mouse IgG1 control demonstrated no inhibitory activity (Fig. 1C). 3E2 could also block SEB-induced activation of human T cells with the highest potency. Furthermore, we analyzed the affinity of mAb 3E2 when binding to SEB on the surface plasmon resonance (SPR)-chip (Fig. 1D). 3E2 was found to bind SEB with very high affinity ($K_d = 5.787 \times 10^{-10}$).

**Overall structure of the SEB-3E2 Fab complex**

SEB achieves its toxicity by interacting with TCRs and MHC class II molecules. To elucidate the mechanism of SEB inhibition through the neutralizing antibody 3E2, we determined the crystal structure of the SEB–3E2 Fab complex using the molecular replacement method and refined it to 3.1 Å resolution with a final $R_{work}$ value of 19.6% ($R_{free} = 26.1$%) (Table S1) (PDB code: 3W2D). In the asymmetric unit, one SEB molecule bound with one 3E2 Fab (Fig. 2), which is consistent with the gel filtration results (Fig. S1).

The superimposition of SEB molecules in free form and 3E2 Fab in bound form yielded a root-mean-square deviation of 0.73 Å for the Cα atoms of 236 residues, particularly for the residues on the interface (Fig. S2). There was no distinct overall structural difference between the free and Fab bound forms of the SEB molecule exists. Notably, residues $\text{SEB}_{D101-109}$ and $\text{SEB}_{D55-57}$, which are not in antibody binding interface, are removed from the final model due to their lack of interpretable electron density. We hypothesized that this is the result of a lack of flexibility and loss of antibody binding. Similar to the apo form, the overall fold of the SEB molecule consists of two domains connected by a six-residue extended loop: domain I ($\text{SEB}_{D30-118}$) and domain II ($\text{SEB}_{D127-238}$), which adopt an oligosaccharide-oligonucleotide fold (OB fold) and a microbial SAg-specific fold, respectively. MHC class II molecules are located in domain II, whereas TCR binding sites are located in the connecting region between domain I and domain II (Fig. S3A). In the SEB molecule, the 3E2 Fab interaction surface overlaps with the MHC binding site but does not interact with the TCR binding site (Fig. S3).

The 3E2 Fab molecule presents a canonical Ig fold consisting of four β-barrel domains (Fig. 1). The light chain is composed of residues M1 to C237, which fold into the $\text{VL}$ and $\text{CL}$ domains; by contrast, the heavy chain is composed of residues M1 to C241, which fold into the $\text{VH}$ and $\text{CH}$ domains. T74, which is located in the classical γ-turn in the Ig family, displays a disallowed stereochemical geometry similar to its counterparts in other reported Fab structures.26 Intramolecular disulfide bonds are found in the expected positions for typical Ig Fab molecules: two between $\text{C45-51}$ and $\text{C117-217}$ and two others between $\text{C40-14}$ and $\text{C166-221}$. The elbow angle of 3E2Fab, which is defined as the angle subtended by the two pseudo 2-fold axes relating $\text{VH}$ to $\text{VL}$ and $\text{CH}$ to $\text{CL}$, is ~170° in the SEB-3E2 Fab complex.

**Interface between SEB and 3E2 Fab**

3E2 Fab binds to SEB using a large and highly complementary interface (Fig. 3), which is consistent with the picomolar binding affinity between 3E2 and SEB (Fig. 1D). The total buried surface area between 3E2 Fab and SEB is 1620 Å² [calculated by PISA27], of which the light and heavy chains of 3E2 Fab contribute 40% and 60%, respectively. The interaction is demonstrated by a high shape complementarity value28 of 0.72 (compared with the average shape complementarity value of 0.64–0.68 for antibody-antigen complexes). Comparison between the interfaces of SEB–3E2 Fab and TCR and the MHC binding sites on SEB suggested that the 3E2 binding site partially occupies the MHC class II molecule binding site, thus allowing 3E2 to inhibit SEB function effectively (Fig. S3).

According to the interaction between the heavy and light chains of 3E2, the 3E2 epitope on SEB could be divided into three regions: region A is formed by the residues in the $\beta3–\beta4$ linking region of SEB domain I and the residues of the heavy chain in 3E2 Fab; region B conducts the interaction between the loop region connecting $\beta1–\beta2$ of SEB and residues in the light chain of 3E2 Fab; and region C, containing $\text{SEB}_{Q43, Y46, F47, K71, K78, K98}$, bonds with both heavy chains and light chains (Fig. 2; Fig. S4). The SEB–3E2 Fab complex is stabilized by 10 hydrogen bonds, two salt bridges, and a number of van der Waals bonds (Table S2). The side chains of $\text{SEB}_{Y46, F47, K71, D72}$, which are located in region C, insert into the deep groove formed by the heavy and light chains of the 3E2 Fab. Particularly, the side chain of $\text{SEB}_{Y46}$ has the deepest insertion into the binding hole. This observation is consistent with the specificity of 3E2 to SEB and denotes $\text{SEB}_{Y46}$ and $\text{SEB}_{K71}$ might play an important role in the interaction between 3E2 and SEB.

**Mutagenesis studies on the neutralizing sites**

---

Figure 2. Overall structure of SEB–3E2 Fab showing the neutralizing epitope. Ribbon diagram (A) and surface representation (B) of the SEB–3E2 Fab complex structure. The SEB, heavy chain, and light chain of 3E2 Fab are shown in green, salmon, and yellow, respectively. The constant and variable regions of 3E2 Fab are labeled accordingly. The residues included in the antigen–antibody interface are shown in red, blue, and green as they are located in the SEB, heavy chain, and light chain, respectively.
In the SEB–3E2 Fab complex structure, a subset of SEB amino acids located on the interface were identified to bind to 3E2 Fab, including SEBQ43, SEBF47, SEBF68, SEBKF71, SEBD72, SEBD75, SEBK69, SEBKF76, SEBK78, SEBK80, SEBK98, and SEBN218.

To determine the key amino acids that could be involved in the epitope structure, we generated a series of substitutions of SEB by replacing the interacting residues with glycine (Table 1) based on structural information. The binding of 3E2 to the mutated proteins and to wild-type (WT) SEB were compared by western blot analysis and ELISA (Fig. 4). Substituting either the SEBY46 or SEBK71 residue with glycine residues dramatically reduced SEB’s capacity to bind with 3E2. Moreover, residue SEBD75 was shown to play a role in the binding of 3E2, based on a reduction in its binding ability. Furthermore, the combinations of SEB43G47 and SEB71G76 were also found to have defective binding capabilities with 3E2. Incubation with PBS control resulted in no detectable response. These results suggest that residues SEB Y46 and SEB K71 play pivotal roles in the interaction with 3E2, which is consistent with the structural information (Fig. 5A).

To further investigate the effect of amino acids side chains on binding affinity between SEB and 3E2, we constructed several point mutations on SEB Y46 and SEB K71. These point mutants were evaluated by ELISA (Fig. 5B) and SPR assay (Fig. 5C–F) for their binding affinities to 3E2. The kinetic binding constants were summarized in Table 2. Substitution of leucine or phenylalanine at SEB Y46 (Y46L or Y46F) resulted in an affinity for 3E2 that was 4- to 60-fold lower. The mutant SEB Y46E resulted in complete loss of binding ability. These results suggested that both the hydrophobic side chain and the hydroxyl group are essential for the function of SEB Y46 in the interaction between 3E2 and SEB.

The same phenomenon was observed in the mutation SEB K71. The mutant SEB K71N resulted in a 2800-fold reduction in affinity, and the SEB K71Q mutant could not bind 3E2 to the same extent as the WT. In contrast, the mutant SEB K71R had no effect on 3E2.
binding, indicating that the positively charged characteristics of the SEB K71 side chain play a critical role in 3E2 binding.

To further investigate the role of these two residues in SEB-induced T cell activation, we measured the proliferative responses of PBMCs to each mutant and WT SEB by analyzing [³H] thymidine uptake (Fig. S5). The WT SEB molecules induced a proliferative response of PBMCs in a concentration-dependent manner. The proliferative responses of PBMCs treated with SEBY46, SEBK71, SEBY46K71, and SEBK71G76 mutants were found to be significantly lower than those of PBMCs treated with WT SEB. The PBMC proliferation induced by mutant SEBD75G was only ~50% of that induced by WT SEB (Fig. S5A). Furthermore, we used a cytokine secretion assay to investigate the ability of WT SEB and mutants to induce cytokine secretion from PBMCs directly. The stimulatory activity of the cytokine secretion of SEB was not completely reduced by the SEBD75G modification alone; however, SEBY46, SEBK71, and the combination of SEBY46 and SEBK71G modifications drastically reduced the secretion of inflammatory cytokines, such as TNF and IFN-γ (Fig. S5B). These results suggest that SEBY46 and SEBK71 play crucial roles in the biological function of SEB, but that SEBD75 is involved in SEB-induced T cell activation. The mechanism of 3E2’s neutralizing function is to block the interaction between SEB and MHC class II molecules through binding with the key residues in SEB function domain.

Type diversity between SAgs on the SEB binding site

SEA, SEB, and SEC are the most important SAgs secreted by S. aureus and have high sequence and structural homologies (1). 3E2 shows high binding affinity and neutralizing activity to SEB; however, it does not bind to SEA and SEC (Fig. 4A). To further understand the specificity of the mAb 3E2 toward SAgs (i.e., SEA, SEB, and SEC), we analyzed the key positions and residues, e.g., glutamine (Q), tyrosine (Y), lysine (K) glutamic acid (E), that affect the molecular interaction.

In the 3E2 epitope, SEBY46 plays an essential role for the interaction, but the same position in SEA is a glutamine residue (Fig. 6). By contrast, the lysine residue at the SEBK71 position is found in both SEA and SEB. Mutagenesis analysis revealed that the SEBY46 mutation completely eliminates binding with 3E2. These findings suggest that SEBY46 accounts for the specific recognition of SEB, but not SEA, by 3E2. Moreover, neither SEBY46 nor SEBK71 are conserved in SEC2 (Fig. 6). In SEC2, an alanine residue and a glutamic acid residue substitute the Y46 and K71 residues in SEB, respectively. The SEBY46A and SEBK71E mutants both show defective binding affinities to 3E2, suggesting that both sites play a role in 3E2 interaction. These observations led us to hypothesize that the residues in the amino acids SEBY46 and SEBK71 are essential to differentiating between the three types of SAgs secreted by S. aureus.

To validate this hypothesis, we generated four constructs by swapping amino acids SEBY46 and SEBK71 on SEA and SEC2 (i.e., SEA Q49Y, SEC A46Y, SEC2 E71K, and SEC2 A46Y+E71K). Western blot analysis revealed that the mutant SEB Q49Y dramatically increased its binding activity to 3E2, whereas other substitutions on SEC2 did not show detectable binding with 3E2 (Fig. 7A). Proliferation of PBMCs induced by WT SEB, SEA, SEC2, and their mutants showed that swapping mutation did not affect the toxicities of SEA and SEC2. However, 3E2 could only inhibit WT SEB and SEA Q49Y induced mitogenesis significantly (Fig. 7B).

We also tested serum IFN-γ and TNF levels induced by SEB, SEA, and their mutants, with or without treatment with 3E2, in the TSS mouse model. The results (Fig. 7C) showed that cytokines induced by SEBY46G were much less than those induced by SEB WT and SEA WT; however, the serum cytokines levels following SEA Q49Y treatment were almost the same as those induced by SEA WT. These results suggest that the swapped mutant of SEA retains its T cell stimulating activity in vivo. 3E2 could efficiently inhibit the release of cytokines induced by SEB WT and SEA Q49Y, but had no inhibitory effects on SEBY46G and SEA WT in vivo. SEA Q49 was also the critical residue for 3E2 to distinguish SEA from SEB. To determine if co-treatment with 3E2 results in a significantly enhanced survival of mice pre-treated with SEB WT or SEA Q73Y, survival was monitored every 6h after treatment for 72h. Mice treated with SEB Q49Y died of TSS during the 3-d period, suggesting that this mutant does not affect the toxicity of SEA. By contrast, mice co-treated with SEA Q49Y and 3E2 survived (Fig. 7D). Taken together, these facts suggest that SEBY46 plays a crucial role in SEB activity and 3E2’s specific recognition of it.

Discussion

SEB is a SAg secreted by S. aureus that is known to cause food poisoning and other fatal diseases by inducing high levels of pro-inflammatory cytokines. These cytokines are released from CD4+ T cells and MHC class II APCs, which are activated through binding of SEB to both MHC class II molecules and
specific TCR Vβ chains. To establish the potential for treating SEB-induced diseases and further understand its inhibitory mechanism, we identified a neutralizing mAb with subnanomolar binding affinity and determined its crystal structure in complex with SEB.

The complex structure of SEB–3E2 Fab revealed a neutralizing conformational epitope on SEB that partially occupied the MHC class II molecule binding site, suggesting that 3E2 functions to block the interaction between SEB and MHC class II molecules. Although the TCR and MHC class II binding sites are adjacent on the surface of SEB and both are crucial for the toxicity of SEB, the key residues (SEBY46 and SEBK71) for 3E2 binding are located in the region of the MHC class II molecule binding site, distal to the TCR binding cleft. This suggests that binding of SEB to MHC class II molecules, but not to TCRs, would be affected by the relevant mutations.

In addition, 3E2 specifically recognizes the MHC class II molecule binding site of SEB. Mutational analysis demonstrated that SEBY46 and SEBK71 are key residues for 3E2 recognition and neutralization. Notably, the antibody 3E2 specifically neutralizes SEB, but not SEA and SEC. To understand how 3E2 discriminates between different SAgS, we compared the sequences of the 3E2

| Mutant | Position | Target Residue | Primer Sequence |
|--------|----------|----------------|-----------------|
| SEB Q43G | Gln43 | Gly | sense: 5′-atcgaccGGAgatcttgacttc-3′ |
| SEB F44G | Phe44 | Gly | sense: 5′-GAGTACAGGATCCGGTCA-3′ |
| SEB L45G | Leu45 | Gly | sense: 5′- Gaccagttcgtacttc-3′ |
| SEB Y46G | Tyr46 | Gly | sense: 5′- CAGTGCAAACACCGGAC-3′ |
| SEB F47G | Phe47 | Gly | sense: 5′- Cagttcgtagagacctgatc-3′ |
| SEB 43G47 | 43QFLYF47 | 43GGGGG47 | sense: 5′-GTAGATCAGGGTCCTCCTCAACTCGTGATGGATT-3′ |
| SEB K71G | Lys71 | Gly | sense: 5′-atcgaccggaggtggagacctgatctac-3′ |
| SEB D72G | Asp72 | Gly | sense: 5′-TGAGCTACAGGGTCCTCCTCAACTCGTGATGGATT-3′ |
| SEB L73G | Leu73 | Gly | sense: 5′-TTTGTACAGGACATCCCTTCTTGGTTTTA-3′ |
| SEB A74G | Ala74 | Gly | sense: 5′-TTTGTACAGGACATCCCTTCTTGGTTTTA-3′ |
| SEB D75G | Asp75 | Gly | sense: 5′-TTTGTACAGGACATCCCTTCTTGGTTTTA-3′ |
| SEB K76G | Lys76 | Gly | sense: 5′-TTTGTACAGGACATCCCTTCTTGGTTTTA-3′ |
| SEB 71G76 | 71KDLADK76 | 71GGGGGG76 | sense: 5′-TTTGTACAGGACATCCCTTCTTGGTTTTA-3′ |
| SEB Q49Y | Gln49 | Tyr | sense: 5′-agtttcgtTACacaccatctc-3′ |
| SEC A46Y | Ala46 | Tyr | sense: 5′-AGATGTGATGAGTGAGCCAGAATC-3′ |
| SEC2 E71K | Glu71 | Lys | sense: 5′-AGCAGGTCTTTGTACCCGAG-3′ |
| SEC2 A46Y E71K | Ala46 & Glu71 | Tyr & Lys | SEC2 A46Y & SEC2 E71K primer |
binding sites on SEA, SEB, and SEC and swapped the distinct residues to examine the binding ability of 3E2. We found that SEBY46 is essential for 3E2 to distinguish SBE from SEA. The results presented here may assist the design and development of specific novel inhibitors for SEB.

**Materials and Methods**

**Ethics statements**

Mice were purchased from the Animal Center of Chinese Academy of Sciences and maintained under pathogen-free conditions. All animal experimental procedures were performed in strict accordance with the guidelines of the Animal Experiment Committee of the International Joint Cancer Institute, and were approved by the Animal Experiment Committee of the International Joint Cancer Institute.

Blood was collected from healthy human volunteers (anonymous) at the International Joint Cancer Institute for the preparation of PBMCs, following written consent in accordance with the Institutional Review Board (IRB) guidelines. The International Joint Cancer Institute IRB approved protocol Hu20110217 for the purpose of the studies described here.

**Construction, expression, and purification of recombinant SEB, SEA, SEC2 and their mutants**

The gene encoding SEB (S407030), SEA (ATCC 29293) and SEC2 (FRI 1230) was chemically synthesized by Sangon and then subcloned into expression vector pGEX-4T-2. SEB, SEA and SEC2 mutants were constructed using QuikChang II Site-Directed Mutagenesis Kit (Stratagene) according to the instructions of the manufacturer. The PCR primer sequences for each mutant are listed in Table 1. The recombinant SAgs were expressed in *Escherichia coli* BL21 (DE3) under the induction of isopropyl-β-d-thiogalactopyranoside. The expressed product was purified using a B-PER GST Fusion Protein Purification Kit (Pierce). The GST fusion sequence was cleaved from the enterokinase recognition sequence DDDDK by enterokinase (Sino Biological Inc.). Digested proteins were dialyzed against 10 mM Tris–HCl (pH 7.5) before being applied to a Superdex-75 column and eluted in 150 mM NaCl. Fractions containing SEB were collected and dialyzed against 10 mM TRIS-HCl (pH 7.5).

**Immunization and generation of anti-SEB mAbs**

BALB/c mice were primed by intraperitoneal injection of 10 μg of SEB emulsified in complete Freund’s adjuvant and boosted once or twice after a 21-d interval by intraperitoneal...
injection of 20 μg of native SEB in 0.5 ml of phosphate-buffered saline. Immunized mice were killed 3 d after secondary or tertiary immunization, and hybridomas were derived and processed as previously described. 29

Immunized mice were killed 3 d after secondary or tertiary injection of 20 μM of 20 mM EDTA, 0.005% Surfactant p20) to a final concentration of 3E2 Fab protein was separated from Fc fragments by protein A affinity chromatography. (GE Healthcare). Further purification was achieved through cationic exchange chromatography and gel filtration on a Superdex-200 column (GE Healthcare).

**Cell proliferation assays**

Human PBMC suspensions (5 × 10⁶ cells/ml) were distributed in 100 μl aliquots to the wells of 96-well flat-bottom plates (5 × 10⁶ cells/well) containing 50 μl of various concentrations of SEB and 50 μl of the indicated concentration of anti-SEB antibody. Each condition was tested in triplicate. The cells were incubated at 37 °C in 75% CO₂ for 48 h and then pulsed with 1 μCi of [³H]thymidine (Amersham-GE Healthcare) per well, incubated for 18 h more, and then harvested onto glass fiber filter strips with a PhD cell harvester (Brandel). The incorporated radioactivity was measured by liquid scintillation counting.

**Cytokine detection**

Human PBMCs were adjusted to 1 × 10⁶ per ml. One hundred microliters of the cell suspension was seeded in a 96-well microplate and then incubated in DMEM at 37 °C in 10% CO₂ with the indicated concentrations of SEB or SEB and antibody. After 24 h of incubation, supernatants were collected and IFN-γ and TNF were measured by ELISA using a sandwich assay involving capture and detection anti-IFN-γ or anti-TNF antibodies (BD PharMingen).

**Surface plasmon resonance assay**

The kinetic parameters of anti-SEB antibodies for SEB were determined using Biacore T100 (Biacore AB). Protein A was immobilized on a CM5 sensor chip using an amine coupling kit according to the manufacturer (Biacore AB). Purified anti-SEB antibody was diluted with running buffer (BIA-certified HBS buffer: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant p20) to a final concentration of 20 μg/ml and applied onto the CM5 chip at a flow rate of 30 μl/min. The CM5 sensor chip was washed with running buffer and recombinant SEB (or SEB mutants) was applied at a flow rate of 30 μl/min. As control, each sample was passed over a reference flow cell containing no ligand (protein A). All binding experiments were performed at 25 °C. The sensor chip could be regenerated with three washes of 3 μl of 20 mM glycine (pH 2.0). The kinetic rates of IgG-SEB binding were derived by globally fitting the Langmuir (1:1) model to the family of association and dissociation curves collected at different ligand concentrations, using BIAevaluation 2.01 software provided by the manufacturer.

**Table 2. Binding affinity of SEB and relevant mutants with 3E2**

|          | Kᵣ (M)     | Kᵣ⁻¹ (M⁻¹s⁻¹) | Kᵣ⁻¹ (s⁻¹) |
|----------|------------|----------------|-------------|
| WT       | 5.787 × 10⁻¹⁰ | 1.068 × 10⁴      | 6.183 × 10⁻⁶ |
| Y46F     | 2.553 × 10⁻⁹  | 2.691 × 10⁴      | 6.871 × 10⁻⁵ |
| Y46L     | 3.144 × 10⁻⁸  | 6.656 × 10⁵      | 2.092 × 10⁴  |
| Y46E     | N/A         | N/A             | N/A         |
| K71R     | 2.810 × 10⁻¹⁰ | 4.381 × 10⁴      | 1.231 × 10⁻⁵ |
| K71N     | 1.419 × 10⁻⁶  | 2.014 × 10⁵      | 2.857 × 10⁻⁴ |
| K71Q     | N/A         | N/A             | N/A         |

*N/A* indicates that the binding affinity was not measured.

©2014 Landes Bioscience. Do not distribute.
crystals were obtained with a reservoir solution containing 150 mM ammonium iodide, 15% PEG 3350, 250 mM ammonium sulfate, and 25 mM Bis-Tris (pH 5.5), and their final dimensions were 30 x 30 x 90 µm³ within 2 mo. The crystals were cryo-protected by soaking in a cryo-protectant consisting of the reservoir solution with additional 10% (v/v) glycol. The cryo-protected crystals were subsequently flash-cooled in liquid nitrogen and then transferred into a dry nitrogen stream at 100 K for X-ray data collection.

**X-ray data collection, processing, and structure determination**

Diffraction data for SEB–3E2 Fab complex crystals were collected at best to 3.1 Å at 100 K using an ADSC Q315 CCD detector on beamline BL5A at Photon Factory. The data sets for complex crystals were indexed, integrated, and scaled using the HKL2000 package. The crystals belong to space group \( P \) with cell parameters \( a = 40.6 \) Å, \( b = 109.3 \) Å, \( c = 86.23 \) Å, and \( d = 96.1^\circ \). The molecular replacement method was used to calculate the phases using the PHASER program. Manual model building and refinement were performed with COOT and PHENIX. Solvent molecules were located from stereochemically reasonable peaks in the \( \sigma \)-weighted \( 2F_\alpha - F_\sigma \) difference Fourier electron density map (1.2 \( \sigma \)). The model geometry was verified using PROCHECK. Structural figures were drawn with PyMOL (www.pymol.org).

Neutralizing effects of 3E2 against SEB, SEA, and SEC2, and their mutants in vitro

PBMCs (5 \( \times 10^5 \) cells per well) were plated in triplicate with WT SEB, WT SEA, WT SEC2, or their mutants (0.1 µg/ml), with or without anti-SEB mAb 3E2 (1 µg/ml). 3E2 was pre-incubated with WT SEB, WT SEA, WT SEC2, and their mutants (0.1 µg/ml) and then added to PBMC cultures (10⁶ per well). Cultures were incubated for 24 h and pulse-labeled with [³H]thymidine for 12 h. Data are expressed as the mean ± SE for triplicate wells. The asterisk represents 3E2 that significantly inhibited WT SEB and SEA Q73Y induced mitogenesis vs. stimulated donor cells in the presence of WT SAE, WT SEC2 and their mutants (\( P < 0.01 \); one-way ANOVA). INF-γ and TNF production in sera from mice stimulated with wild type SEB WT, SEA WT and their mutants SEB Y46G, SEA Q49Y alone or in the presence of 3E2. Mice were injected with D-GaIN (20 mg/mouse), lipopolysaccharides (1 mg/mouse), as well as SEB, SEA, and SEA Q49Y (20 µg/mouse; 10 LD₅₀). After 4 h, 3E2 (600 µg/mouse) was injected in the indicated groups. Mice sera were collected and cytokines were detected by ELISA. Results are mean ± SE for triplicate measurements in 6 different donors. (\( * P < 0.01 \); one-way ANOVA)(D) Animal survival was monitored every 6 h for 72 h. Statistical significance was calculated by the log-rank test (\( P < 0.01 \); n = 10).
and V values of less than 0.05 were considered statistically significant.

Acknowledgments

This work was supported by the Ministry of Science and Technology 973 (Grant Nos. 2010CB833605, 2013CB911003, and 2010CB735605), National Natural Science Foundation of China (Grant Nos. 31170678 and 31000332), Key Projects in the Tianjin Science and Technology Pillar Program (Grant No. 10ZCKFSY08800), and 2011 Science and Technology Innovation Fund (Grant No. 11C26211203971). We thank the staff of Photon Factory for their generous help with diffraction data collection.

References

1. Li H, Llera A, Tsuchiya D, Leder L, Yi, Shlomchik M, Karjalainen K, Mariuzza RA. Three-dimensional structure of the complex between T cell receptor beta chain and the superantigen staphylococcal enterotoxin B. Immunity 1998; 9:807-16; PMID:981971; http://dx.doi.org/10.1016/S1074-7613(00)80646-9

2. Li H, Llera A, Malchiodi EL, Mariuzza RA. The structural basis of T cell activation by superantigens. Annu Rev Immunol 1999; 17:495-66; PMID:10358765; http://dx.doi.org/10.1146/annurev.immunol.17.1.435

3. Bohach GA, Fast DJ, Nelson RD, Schlievert PM. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Crit Rev Microbiol 1990; 17:251-72; PMID:2206394; http://dx.doi.org/10.3109/10408418909105728

4. Buonpane RA, Chuchill HR, Moza B, Sundberg EJ, Peterson ML, Schlievert PM, Kranz DM. Neutralization of staphylococcal enterotoxin B by soluble, high-affinity receptor antagonists. Nat Med 2007; 13:725-9; PMID:17515896; http://dx.doi.org/10.1038/nm1584

5. Burnett JC, Henschel EA, Schmaljohn AL, Bavari S. The evolving field of biodefence: therapeutic developments and diagnostics. Nat Rev Drug Discov 2005; 4:281-97; PMID:15803939; http://dx.doi.org/10.1038/nrd1694

6. Earhart CA, Vath GM, Roggiani M, Schlievert PM, Ohlendorf DH. Structure of streptococcal pyrogenic exotoxin A reveals a novel metal cluster. Protein Sci 2000; 9:1845-51; PMID:11045650; http://dx.doi.org/10.1110/ps.9.9.1847

7. Swaminathan S, Furey W, Pletcher J, Sax M. Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature 1992; 359:801-6; PMID:1346058; http://dx.doi.org/10.1038/359801a0

8. Papageorgiou AC, Acharya KR, Shapiro R, Passalacqua EF, Brehm BD, Tramier HS. Crystal structure of the superantigen enterotoxin C2 from Staphylococcus aureus reveals a zinc-binding site. Structure 1995; 3:769-79; PMID:7582894; http://dx.doi.org/10.1016/S0909-4401(01)00212-X

9. Acharya KR, Passalacqua EF, Jones EY, Harlos K, Stuart DI, Brehm RD, Tramier HS. Structural basis of superantigen action inferred from crystal structure of toxic shock syndrome toxin-1. Nature 1994; 367:94-7; PMID:8107781; http://dx.doi.org/10.1038/36794a0

10. Prasad GS, Earhart CA, Murray DL, Novick RP, Schlievert PM, Ohlendorf DH. Structure of toxic shock syndrome toxin 1. Biochemistry 1993; 32:13761-6; PMID:2861850; http://dx.doi.org/10.1021/bi01021a001

11. Jardetzky TS, Brown JH, Gorga JC, Stern LJ, Urban RG, Chi YJ, Strafocher C, Strominger J, Wiley DC. Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. Nature 1994; 367:711-8; PMID:8152483; http://dx.doi.org/10.1038/367711a0

12. Kappler JW, Herman A, Clements J, Marrack P. Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. J Exp Med 1992; 175:387-96; PMID:1370682; http://dx.doi.org/10.1084/jem.175.2.387

13. Faulkner L, Cooper A, Fantino C, Altmann DM, Stiskandad S. The mechanism of superantigen-mediated toxic shock: not a simple Th1 cytokine storm. J Immunol 2005; 175:6870-7; PMID:16272345

14. Miethke T, Wahl C, Heeg K, Echtenacher B, Krammer PH, Wagner H. T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor. J Exp Med 1992; 175:91-8; PMID:1730929; http://dx.doi.org/10.1084/jem.175.1.91

15. Gordon GM, Du W. Conserved RB functions in development and tumor suppression. Protein Cell 2011; 2:864-78; PMID:22180866; http://dx.doi.org/10.1007/s13238-011-1117-x

16. Kaempfer R. Peptide antagonists of superantigens. Mol Divers 2004; 8:115-29; PMID:15201962; http://dx.doi.org/10.1023/B:MOLE.0000025654.04427.44

17. Rajagopalan G, Sen MM, David CS. In vitro and in vivo evaluation of staphylococcal superantigenic peptide antagonists. Infect Immun 2004; 72:673-7; PMID:1508183; http://dx.doi.org/10.1128/IAI.72.673-6737.2004

18. Darenberg J, Soderquist B, Normark BH, Norby-Teglund A. Differences in potency of intravenous polyspecific immunoglobulin G against streptococcal and staphylococcal superantigens: implications for therapy of toxic shock syndrome. Clin Infect Dis 2004; 38:836-42; PMID:14999628; http://dx.doi.org/10.1086/381979

19. Bavari S, Ulish RG, LeCrae DR. Cross-reactive antibodies prevent the lethal effects of Staphylococcus aureus superantigens. J Infect Dis 1999; 180:1365-9; PMID:10479174; http://dx.doi.org/10.1086/354977

20. Hamad AR, Herman A, Marrack P, Kappler JW. Monoclonal antibodies defining functional sites on the toxin superantigen staphylococcal enterotoxin B. J Exp Med 1994; 180:605-21; PMID:7579243; http://dx.doi.org/10.1083/jem.180.2.615

21. Varshney AK, Wang X, Cook E, Dutta K, Scharff MD, Goger MJ, Fries BC. Generation, characterization, and epitope mapping of neutralizing and protective monoclonal antibodies against staphylococcal enterotoxin B-induced lethal shock. J Biol Chem 2011; 286:9737-47; PMID:2123204; http://dx.doi.org/10.1074/jbc.M110.212407

22. Tilahun ME, Rajagopalan G, Shah-Mahoney N, Lawlor RG, Tilahun AY, Xie C, Natarajan K, Margulies DH, Ratiner DM, Osborne BA, et al. Potent neutralization of staphylococcal enterotoxin B by synergistic action of chimeric antibodies. Infect Immun 2010; 78:2801-11; PMID:20308394; http://dx.doi.org/10.1128/IAI.01121-09

23. Pang LT, Kum WW, Chow AW. Inhibition of staphylococcal enterotoxin B-induced lymphocyte proliferation and tumor necrosis factor alpha secretion by MABs, an anti-toxic shock syndrome toxin 1 monoclonal antibody. Infect Immun 2000; 68:3261-8; PMID:10816471; http://dx.doi.org/10.1128/IAI.68.6.3261-3268.2000

24. Murzin AG. OBLigomerNucleotide oligooxacarboxylic binding)-fold: common structural and functional solution for non-homologous sequences. EMBO J 1993; 12:861-7; PMID:8458342

25. Papageorgiou AC, Tramier HS, Acharya KR. Crystal structure of microbial staphylococcal superantigen enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. J Mol Biol 1998; 277:61-79; PMID:9514739; http://dx.doi.org/10.1006/jmbi.1997.1577

©2014 Landes Bioscience. Do not distribute.
26. Al-Lazikani B, Lesk AM, Chothia C. Standard conformations for the canonical structures of immunoglobulins. J Mol Biol 1997; 273:927-48; PMID:9367782; http://dx.doi.org/10.1006/jmbi.1997.1354

27. Krissinel E. Crystal contacts as nature’s docking solutions. J Comput Chem 2010; 31:133-43; PMID:19423996; http://dx.doi.org/10.1002/jcc.21303

28. Lawrence MC, Colman PM. Shape complementarity at protein/protein interfaces. J Mol Biol 1993; 234:946-50; PMID:8263940; http://dx.doi.org/10.1006/jmbi.1993.1648

29. Goldsby RA. in Nucleic acid and monoclonal antibody probes, eds. Swaminathan, B. & Prakash, G. (Marcel-Dekker, New York, NY), 1989; pp. 367.

30. Chen Q, Wang Q, Xiong L, Lou Z. A structural view of the conserved domain of rice stress-responsive NAC1. Protein Cell 2011; 2:55-63; PMID:21337010; http://dx.doi.org/10.1007/s13238-011-1010-9

31. Orwinowski Z, Minor W. in Macromolecular Crystallography, part A, eds. Carter Jr., C. W. & Sweet, R. M. (Academic Press, New York), 1997; Vol. 276, pp. 307-326.

32. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J Appl Crystallogr 2007; 40:658-74; PMID:19461840; http://dx.doi.org/10.1107/S002188980721206

33. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 2004; 60:2126-32; PMID:15572765; http://dx.doi.org/10.1107/S090744490401958

34. Adams PD, Grosse-Kunstleve RW, Hung LW, Ioerger TR, McCoy AJ, Moriarty NW, Read RJ, Sacchettini JC, Sauter NK, Terwilliger TC. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr D Biol Crystallogr 2002; 58:1948-54; PMID:12393927; http://dx.doi.org/10.1107/S0907444902016657

35. Laskowski R, MacArthur M, Moss D, Thornton J. PROCHECK: a program to check the stereochemical quality of protein structures. J Appl Cryst 1993; 26:283-91; http://dx.doi.org/10.1107/S0021889892009944

36. Blank C, Luz A, Bendigs S, Erdmann A, Wagner H, Heeg K. Superantigen and endotoxin synergize in the induction of lethal shock. Eur J Immunol 1997; 27:825-33; PMID:9130631; http://dx.doi.org/10.1002/eji.1830270405

37. Xuan C, Shi Y, Qi J, Zhang W, Xiao H, Gao GF. Structural vaccinology: structure-based design of influenza A virus hemagglutinin subtype-specific subunit vaccines. Protein Cell 2011; 2:997-1005; PMID:22231357; http://dx.doi.org/10.1007/s13238-011-1134-y