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High Affinity T Cell Receptors from Yeast Display Libraries Block T Cell Activation by Superantigens

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The αβ T cell receptor (TCR) can be triggered by a class of ligands called superantigens. Enterotoxins secreted by bacteria act as superantigens by simultaneously binding to an MHC class II molecule on an antigen-presenting cell and to a TCR β-chain, thereby causing activation of the T cell. The cross-reactivity of enterotoxins with different Vβ regions can lead to stimulation of a large fraction of T cells. To understand the molecular details of TCR-enterotoxin interactions and to generate potential antagonists of these serious hyperimmune reactions, we engineered soluble TCR mutants with improved affinity for staphylococcal enterotoxin C3 (SEC3). A library of randomly mutated, single-chain TCRs (Vβ-linker-Vα) were expressed as fusions to the Aga2p protein on the surface of yeast cells. Mutants were selected by flow cytometric cell sorting with a fluorescent-labeled SEC3. Various mutations were identified, primarily in Vβ residues that are located at the TCR:SEC3 interface. The combined mutations created a remodeled SEC3-binding surface and yielded a Vβ domain with an affinity that was increased by 1000-fold ($K_D = 7$ nM). A soluble form of this Vβ mutant was a potent inhibitor of SEC3-mediated T cell activity, suggesting that these engineered proteins may be useful as antagonists.

Keywords: T cell activity; antagonists; *Staphylococcus aureus*; yeast surface display; directed evolution

Introduction

The αβ T cell receptor (TCR) on the surface of T cells is responsible for the specific recognition of antigenic peptides bound to proteins encoded by the major histocompatibility complex (MHC). TCRs are similar in structure to antibodies; the α and β subunits are composed of constant and variable domains and each Vα and Vβ domain contains several loops of highly variable sequence, including three complementarity determining regions (CDR1-3) and a fourth hypervariable loop (HV4). Together, these CDR and HV loops form the surface of the TCR that binds the peptide-MHC antigen complex.[1-3]

Over a decade ago, TCRs were shown to bind another class of ligands, called superantigens (SAgs) because they stimulated a large fraction of T cells.[4] Enterotoxins secreted by the Gram-positive bacterium *Staphylococcus aureus* are among the most studied of the SAgs.[5] T cells activated by binding a staphylococcal enterotoxin (SE) release cytokines such as TNFα, leading to inflammatory reactions associated with food poisoning, toxic shock syndrome, and septic shock.[6,7] SEs represent a family of related proteins, including SEB and SEC3, that bind to both a TCR Vβ region and an MHC class II molecule.[5,8] Recent X-ray crystallographic structures of a Vβ-SEC3 complex, a SEB-class II MHC complex and a modeled VαVβ-SEC3-class II ternary complex, have shown the key features of these interactions.[9-12] Most of the contacts between TCR and SEC3 resided in CDR2 and HV4 of the β-chain. A mutagenesis study

Abbreviations used: FR, framework region; HV, hypervariable region; MFU, mean fluorescent units; SPR, surface plasmon resonance; SAv-PE, streptavidin-phycoerythrin; PCR, polymerase chain reaction; MHC, major histocompatibility complex; CDR, complementarity determining regions; pMHC, peptide-MHC complex; SAg, superantigen; scTCR, single-chain TCR; SE, *Staphylococcus aureus* enterotoxin; trp, tryptophan; V, variable; wt, wild-type; RU, response unit.

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supported the view that the CDR2 loop contributed the majority of binding energy to the interaction.[13] The model and mutagenesis studies also suggested that the Vα domain, and CDR2α in particular, could stabilize the entire ternary complex by contacting the class II MHC helices.[11]

Despite the potent biological effects of SAgs, it is interesting that the intrinsic binding affinities of the TCR for SEs are quite low, with K_D values in the range of 1 to 100 μM.[11,14-16] These affinities are actually quite similar to those observed for the interaction of TCRs with their conventional antigens, peptide-MHC complexes.[17,18] While these relatively low affinities are sufficient to stimulate T cell activity, they have made biochemical studies of TCR-ligand interactions difficult. In addition, the use of soluble TCRs as antagonists, in line with a strategy that has proven successful with other receptor systems[19,20] would be virtually impossible at these low affinities.

A standard method for improving the affinity of peptides or some proteins, including antibodies, has been phage display.[21] Efforts to display TCRs on the surface of phage have, with few exceptions,[22] been unsuccessful. However, a yeast display system[23] provided a method for engineering mutant TCRs that were stabilized and maintained binding to specific peptide-MHC and SE ligands.[24-26] More recently, the yeast display system was used to isolate TCR mutants in CDR3α that exhibited a 100-fold higher affinity for a peptide-MHC complex.[27]

We show here that the yeast display system can also be used to further understand the molecular basis of TCR-SEC3 interactions and to evolve TCRs with higher affinity for this important class of antigens. Although our selection system was initially applied to a library of random mutants of the full-length single-chain TCR (Vβ-linker-Vα), we discovered that the minimal SEC3 binding domain, the Vβ8 region, was strongly selected because of its higher level of surface expreession. An additional round of mutagenesis followed by flow cytometric cell sorting yielded isolates with additional, multiple mutations. When nine of the mutations were combined, the Vβ mutant (mL2.1/A52V) had an improvement in SEC3 binding affinity of ~1000-fold ($K_D = 7$ nM), compared to the wild-type Vβ. Several of the mutations resided in an energetic ''hot spot'' at the Vβ:SEC3 interface and others were in surface residues that were more distal to this region. This finding emphasizes the possible advantage of using mutagenesis strategies that explore sequence space across the entire interface area. Finally, we show that a soluble form of the high affinity Vβ was able to completely inhibit SEC3-mediated T cell activation, indicating the potential of these engineered TCR domains as antagonists of enterotoxin-mediated diseases.

Results

Yeast display and selection of mutant TCRs

The T cell clone called 2C expresses an αβ heterodimer that recognizes various ligands, including the alloantigenic peptide-MHC (p2Ca/L^d), a self peptide-MHC (dEV8/K^b), and enterotoxins that bind to the Vβ8.2 region expressed by 2C.[28-30] Hence, clone 2C is stimulated very effectively by SEB and SEC3.[31] Using a panel of single-site mutants of the 2C scTCR expressed in *Escherichia coli*, we recently performed binding studies with an SEC3 variant (called SEC3-1A4) in order to construct an energy map of the Vβ8-SEC3 interaction.[13] SEC3-1A4 had itself been isolated by phage display, selecting mutations in the disulfide loop that yielded higher affinity for the Vβ8.2 region. The higher affinity of the Vβ8-SEC3-1A4 interaction (compared to the Vβ8-SEC3 interaction) provided sufficient affinity to detect reduced binding by some of the single-site mutants.

To ascertain if the scTCR could be engineered for increased SEC3 binding affinity, we exploited a relatively new system called yeast display. We have shown that the 2C scTCR could only be expressed on the surface of yeast, as fusions to Aga2p, when there were selected amino acid substitutions in the TCR that yielded a more stable protein.[24-26] In the present study, a library was generated using error-prone PCR of several stabilized scTCR mutants, including the wild-type scTCR and the mG17E mutant. Approximately 3×10^5 independent transformants, with an average of five mutations per scTCR gene, were expressed on the surface of yeast cells. As the low affinity of wild-type SEC3 ($K_D = 3$ μM) may not have allowed detection of improved mutants, we reasoned that it might be advantageous to use SEC3-1A4 as the initial selecting ligand. In this regard, binding of biotinylated SEC3-1A4, but not biotinylated SEC3, to the yeast displayed-mG17E mutant was detected with streptavidin-phycoerythrin (SAv-PE) and flow cytometry (data not shown). Based on a titration of yeast cells with biotinylated SEC3-1A4, a limiting concentration of SEC3-1A4 (~1 nM) was used to select the library. Yeast cells were subjected to three cycles of flow cytometric cell sorting and the top 2% to 3% most fluorescent cells were collected (round one of mutagenesis and selection; Figure 1).

Plasmids from 14 colonies were rescued and sequenced. Among the 14 isolates, there were six unique sequences (for listing of isolates and frequency, see Materials and Methods). Two mutations, βG17E and βS54N, were present in all six classes of mutants. The βG17E mutation is known to stabilize the scTCR, whereas βS54N is located at the Vβ:SEC3 interface. Residue βS54 may also be important in stabilizing the TCR, as scTCR with the βS54A mutation does not fold properly.[32] In addition to these mutations, eight isolates (representing two of the unique classes)

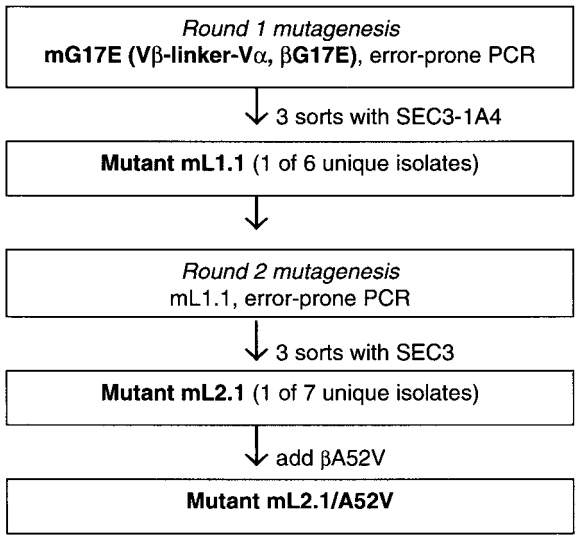

Figure 1. Summary of the yeast display strategy used to generate TCR mutants with improved binding to superantigen. Names of selected mutants are indicated in bold.

contained stop codons that resulted in a truncated Vβ domain only. Analysis of these isolates with an anti-Vβ8 antibody showed that they were expressed at considerably higher surface levels than any of the full length scTCR mutants, including those that expressed both βG17E and βS54N (data not shown). Thus, fusion with the Vα region appears to reduce the surface levels of the displayed protein, perhaps because it folds into a domain structure less efficiently than the Vβ8 region or because it is less stable.[33,34]

In order to further engineer an improved SEC3-binding protein, mL1.1, an isolate from the first round of mutation that contained the two common mutations (βG17E and βS54N) and the Vβ only domain, was used as the starting material for a second round of error-prone PCR mutagenesis. Mutant mL1.1 also contained two other mutations (βT87S and βG96V) that are located more distal to the SEC3 binding site. These mutations may have been carried along with the other mutations and therefore had no effect on SEC3 binding or perhaps they provided some stability to the protein. An mL1.1 (βG17E, βS54N, βT87S and βG96V) library of 2×10^5 independent transformants, containing approximately two mutations per Vβ gene was subjected to flow cytometric sorting with SEC3. SEC3 was used rather than SEC3-1A4 because binding with biotinylated SEC3 could now be detected with the mL1.1 starting Vβ. Based on this binding experiment, a limiting concentration of SEC3 (14 nM) was used to select the mL1.1 library. Yeast cells were subjected to three sorts and the top 1.5% to 2% most fluorescent cells were collected (Figure 1). Plasmids from 14 colonies were rescued and sequenced and seven unique isolates were identified. One of these mutants, mL2.1, contained mutations βK66E, βQ72H, βE80V, and βL81S, that are located at the Vβ8:SEC3 interface[10]. Several of the other isolates contained another mutation, βA52V, that lies within a region of CDR2 (residues 51, 52 and 53) that we had previously identified as the hot spot of SEC3 binding.[13] Thus, mL2.1 (βG17E, βS54N, βK66E, βQ72H, βE80V, βL81S, βT87S and βG96V) and the mL2.1/A52V variant (βG17E, βS54N, βK66E, βQ72H, βE80V, βL81S, βT87S, βG96V and βA52V), in which we introduced the βA52V mutation, were examined further for SEC3 binding affinity.

Characterization of mutant TCRs for binding to SEC3

Mutant TCRs isolated from the selections described above were examined by flow cytometric titrations with SEC3 (Figure 2(a)), SEC3-1A4 (Figure 2(b)), and SEB (data not shown). To compare with the wild-type Vβ8, two additional constructions were included, a Vβ8 region with the original stabilizing mutation βG17E, and a double mutant, βG17E/βL81S. The latter mutation was isolated independently in selections based on thermal stability.[26] It was produced in order to explore if the βL81S mutation might also contribute additional SEC3 binding energy, for example in the mL2.1 isolate. In order to control for possible effects of TCR surface levels on binding to the SEC3 ligands, all of the mutants contained the Vβ8 region alone and a C-termnal c-*myc* epitope tag that could be used to compare surface levels. Two isolates, mG17E/L81S and mL2.1/A52V (βG17E, βS54N, βK66E, βQ72H, βE80V, βL81S, βT87S, βG96V and βA52V), were sub-cloned into a yeast secretion system[25] and the purified proteins were further examined by surface plasmon resonance (SPR) for binding to SEC3 and SEC3-1A4.

Binding titrations of the mutants compared to the stabilized wild-type (mG17E) showed approximately a tenfold increase in SEC3-1A4 binding for the most improved mutant (mL2.1/A52V). In contrast, the binding to wild-type SEC3 showed an almost 1000-fold improvement for mL2.1/A52V. The estimated K_D value from the SEC3 titration was 6 nM for mL2.1/A52V, compared to undetectable binding for mG17E and mG17E/L81S. Previous studies have shown that Vβ8 has an affinity of approximately 5 to 20 μM for SEC3.[13] While each of the possible single-site mutations were not examined individually, the first round mutations βS54N, βT87S, and βG96V yielded an affinity increase of threefold for SEC3-1A4 and tenfold for SEC3. The second round mutations βK66E, βQ72H, and βE80V yielded a further affinity increase of threefold for SEC3-1A4 and tenfold for SEC3. The βA52V mutation appears to have little effect on SEC3-1A4 binding, but yields approximately a threefold increase for SEC3 binding. Interestingly, the mL2.1/A52V mutant also bound to the related enterotoxin SEB, with 500-fold improved affinity

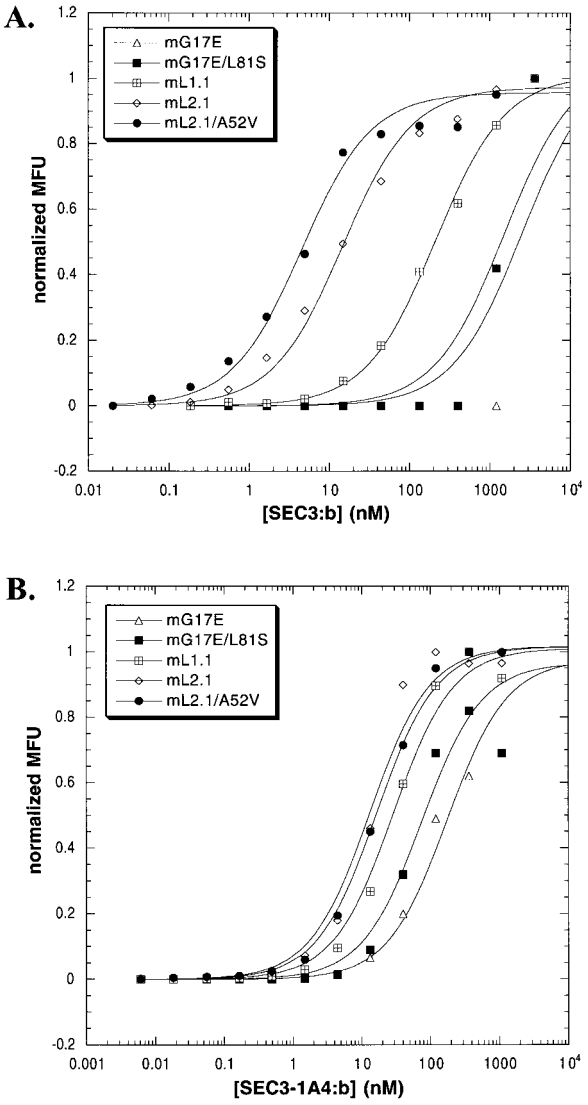

Figure 2. SEC3 and SEC3-1A4 binding of various TCR mutants monitored by flow cytometry. Five Vβ TCR mutants were titrated with various concentrations of either wild-type SEC3:biotin (a) or the higher affinity variant SEC3-1A4:biotin (b) and analyzed by flow cytometry to determine apparent K_D values (Table 1). Apparent K_D values of mutants mG17E and mG17E/L81S for SEC3-1A4:biotin were determined by estimating the maximum SAg binding level (maximum mean fluorescence units, MFU), since binding the highest testable concentration of SEC3-1A4:biotin did not reach saturation. The maximum for these two mutants was calculated using the average of the other three Vβ mutants (which reached saturation at the highest concentration of SEC3-1A4:biotin). The values among these different mutants is likely to be similar as the surface level of the mutants, except for mG17E, was approximately equal based on saturation binding of a mAb to the c-*myc* epitope tag at the C terminus of the Vβ protein. Data were plotted as normalized MFU, calculated by subtracting the background MFU (in the absence of SEC3 but presence of SAv-PE) from the measured raw values and then dividing by the maximum MFU (MFU at saturation).

($K_D \sim 250$ nM, data not shown). SEB shares about 66% amino acid sequence homology with SEC3 and binds with approximately 50-fold lower affinity to wild-type Vβ8.2 (K_D value of Vβ8.2 for SEB ~ 140 μM.[16] Thus, selection for binding to one SAg can yield improved binding to other related SAgs, a finding that may be useful in engineering Vβ proteins as *in vivo* antagonists (see below).

In order to confirm these binding studies, SPR was used with soluble forms of the two proteins mG17E/L81S and mL2.1/A52V. The 6-His-linked proteins were purified by Ni-chromatography followed by size filtration and used for binding to immobilized SEC3 or SEC3-1A4. SPR analysis of the TCR expected to behave like the wild-type Vβ8 (mG17E/L81S) showed that there was not detectable binding to SEC3 at the concentrations tested (serial dilutions of a 4 μM stock, which is below the K_D; Figure 3(a) and data not shown) but that there was binding to the SEC-1A4 variant (Figure 3(b)). The equilibrium binding constant determined for the latter interaction was 282 nM (Table 1). The affinity of this interaction is similar to that between the murine 14.3.d TCRβ chain and SEC3-1A4, measured to be 242 nM using the same immobilized SEC-1A4 sample. This shows that the wild-type Vβ8 (mG17E/L81S), the template used for affinity maturation through mutagenesis, behaves similarly to the previously analyzed TCRβ chain classified as Vβ8.2 [13,15]. In striking contrast, the mL2.1/AV2 Vβ8 mutant bound well to both immobilized SEC3 and SEC3-1A4 (Figure 3(c) and (d), respectively). The K_D values for these interactions based on kinetic data were found to be 7.3 and 2.2 nM, respectively. Affinity values calculated by Scatchard analysis of equilibrium titrations gave similar results, with slightly higher K_D values (10.5 and 4.3 nM, respectively; data not shown). Thus, the SPR results confirmed the relative binding affinities estimated by flow cytometry and they showed that the engineered mL2.1/A52V Vβ domain has an affinity that is increased by approximately 1000-fold for the wild-type SEC3.

Location of mutations in the Vβ8:SEC3 structure

Figure 4(a) shows the location of the nine mutations present in the final Vβ8 mutant, mL2.1/A52V, in the three-dimensional structure of the Vβ8:SEC3 complex.[10] All of the mutations reside on the surface of the Vβ8 molecule. Three of the mutations, βA52V, βS54N, and βQ72H, are positioned at or very near the energetic hot spot identified previously by our laboratory.[13] Eight out of nine of the mutations reside on the same face of the molecule that binds to SEC3 (Figure 4(b)). Four of these mutations, βK66E, βE80V βL81S, and βG17E, also reside in a patch that is at or near the SEC3 interface (although only βK66 is in contact with SEC3 in the crystal structure). From the locations of these two clusters, it appears that a significant fraction of the interface has been remo-

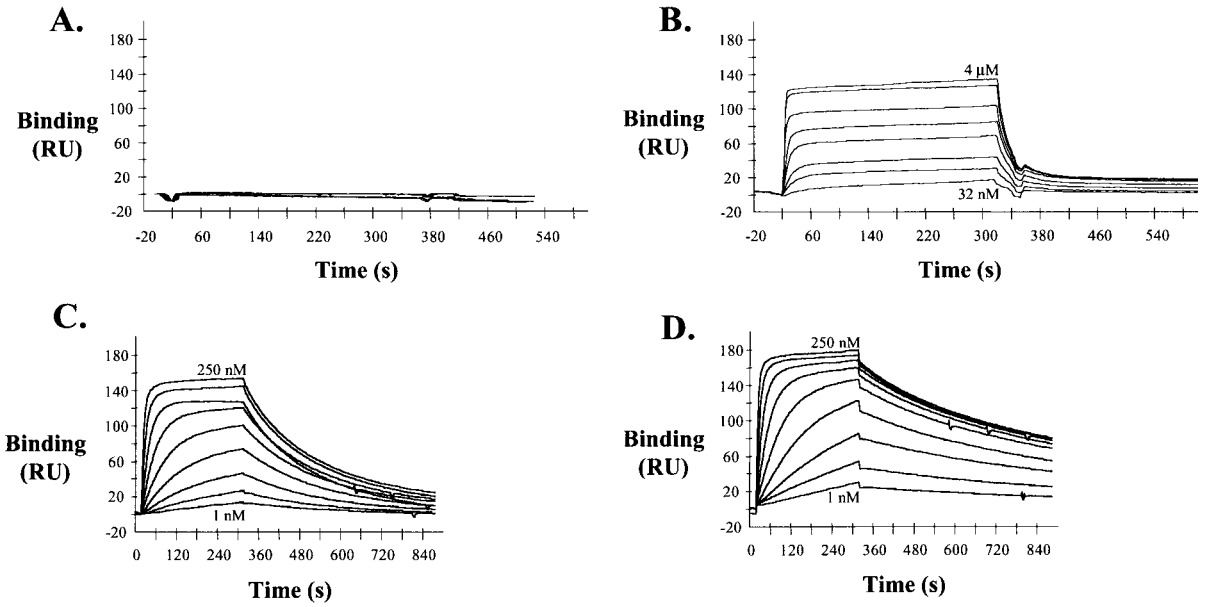

Figure 3. SEC3 binding to two Vβ 2C TCR mutants assayed by surface plasmon resonance (SPR). SPR analysis of mG17E/L81S binding to a blank sensor chip (a) or mutant SEC3-1A4 (b), and mL2.1/A52V binding to wild-type SEC3 (c) or SEC3-1A4 (d). Serial twofold dilutions of mG17E (4 μM stock) or mL2.1/A52V (250 nM stock) were injected at a flow rate of 25 μl/minute over immobilized SEC3 or SEC3-1A4 (500 RU). K_D values (Table 1) were calculated as the ratio of the on and off-rates. RU refers to response units. Mutant mG17E/L81S binding to SEC3 was also undetectable and identical to the sensorgram shown in (a).

deled in order to achieve the overall increase in binding affinity for the SEC3 ligand. This finding is reminiscent of studies with human growth hormone, in which a mutant selected by phage display showed very significant conformational changes at the interface with its receptor, compared to the wild-type.[35] It is unclear why the affinities of the final Vβ8 molecule (mL2.1/A52V) complexed with wild-type SEC3 and SEC3-1A4 are so similar when there is such a large discrepancy (~100-fold) between the parent Vβ8 molecule (mG17E/L81S) complexed with the wild-type and 1A4 variant SEC3 molecules. All of the mutated residues except βQ72H, and possibly βG96V, are located far enough away from the mutated loop of SEC3-1A4 that it is easy to see why the relative affinity increases might be greater for the wild-type SEC3 compared to the mutant SEC3-1A4. Conversely, based on their location one might have expected these mutations to yield equally significant increases in the affinity of mL2.1/A52V for SEC3-1A4 as for SEC3 (i.e. 1000-fold).

Table 1. Binding affinities of various Vβ8.2 mutants for SEC3 and SEC3-1A4

		SEC K_D (nM)		SEC3-1A4 K_D (nM)	
Vβ mutant	Mutations[a]	flow cyt.[b]	SPR[c]	flow cyt.	SPR
mG17E	βG17E	>3600		140 ± 51	
mG17E/L81S	βG17E, β*L81S*	>3600	>4000	58 ± 24	282
mL1.1	βG17E, βS54N, βT87S, βG96V	217 ± 13		27 ± 4	
mL2.1	βG17E, βS54N, βK66E, βQ72H, βE80V, β*L81S*, βT87S, βG96V	20 ± 5		11 ± 4	
mL2.1/A52V	Same as mL2.1, plus βA52V	6 ± 3	7.3	13 ± 7	2.2

[a] Mutations found in five Vβ TCR mutants are listed. Vβ-SEC3 contact residues[7] are underlined and the stability mutation L81S[49] is shown in italics.

[b] Flow cytometry-derived apparent affinities for both wild-type SEC3 (Figure 2(a)) and the mutant SEC3-1A4 (Figure 2(b)) were determined by binding titrations. Samples were washed after incubation with biotinylated-SAg ligand and after incubation with SAv-PE, which may affect the affinity values calculated by this method.

[c] Surface plasmon reonance (SPR) of binding by soluble forms of the two Vβ proteins mG17E/L81S and mL2/A52V to immobilized SEC3 and SEC3-1A4 (Figure 3).

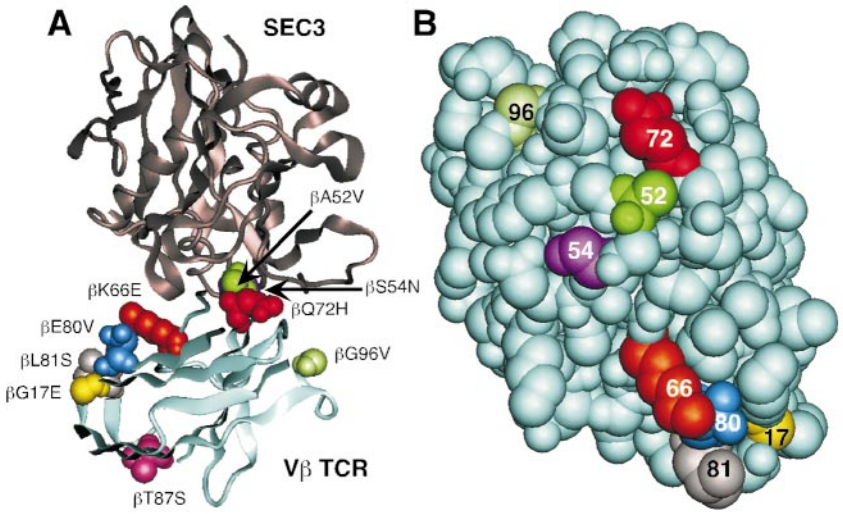

Figure 4. Location of Vβ mutations in the Vβ8-SEC3 complex (RCSB PDB accession code 1JCK). Crystal structure with the positions of the mutated Vβ 2C TCR residues indicated. The program QUANTA (Molecular Simulations) was used to label mutations in the 14.3.d TCR β8.2 chain-SEC3 crystal structure[9] (a). Only the Vβ domain of the TCR is shown with SEC3. A space-filling model of the Vβ domain face that binds to SEC3 is shown with the positions of the mutations labeled (b). Mutant residues in both panels are colored as follows: βG17E, yellow; βA52V, green; βS54N, purple; βK66E, orange; βQ72H, red; βE80V, blue; βL81S, gray; βT87S, pink; βG96V, light green.

Inhibition of SEC3-mediated T cell activity by a soluble Vβ mutant

Higher affinity SEC3-binding proteins have potential use as an antagonist of the T cell activity elicited by the enterotoxin. Soluble forms of the highest affinity mutant, mL2.1/A52V and the wild-type control, mG17E/L81S, were tested in a T cell assay involving the cytotoxic T lymphocyte clone 2C. CTL 2C recognizes and lyses target cells that bear its normal peptide/MHC antigen (peptide/L^d) present on the tumor P815 and also MHC class II bearing target cells that have been incubated with SEC3.[31] In these experiments, the MHC class II-positive target cell Daudi was loaded with ^{51}Cr and then incubated with 2C T cells, SEC3, and various concentrations of purified Vβ proteins (Figure 5(a)). As a control, the target cell P815 was examined; only the full αβ TCR can recognize the peptide/L^d antigen and thus the Vβ domains alone would not specifically block this recognition (Figure 5(b)). Significant inhibition of SEC3-mediated lysis was observed with the mL2.1/A52V protein at 10 nM and above. In contrast, recognition of the peptide/L^d ligand (P815) was not observed, even at the highest concentration of mL2.1/A52V tested, 300 nM. The low affinity protein mG17E/L81S, did not inhibit SEC3-mediated activity at the highest concentration tested (300 nM). The complete inhibition of SEC3-mediated T cell activity suggests that higher affinity Vβ domains could be effective antagonists of T cell-mediated responses.

Discussion

Affinity maturation of antibodies *in vivo* is accomplished through somatic mutation followed by selection processes that operate on B cells, leading to the enrichment of cells that express higher affinity antibody variants. Antibodies have also been selected *in vitro* through genetic engineering and selection techniques such as phage and yeast display that can enrich for yet higher affinity variants.[21,36,37] In contrast, T cell receptors do not undergo *in vivo* somatic mutation or a similar degree of affinity maturation and *in vitro* approaches have, with one exception,[27] not proven successful. Here, we describe the *in vitro* engineering of a scTCR for improved binding to the superantigen SEC3 from *Staphylococcus aureus*. A library of randomly mutated scTCR proteins was displayed on the surface of yeast cells, and mutants with higher affinity for SEC3 were selected using cell sorting. The minimal SEC3-binding domain, Vβ, was identified using this strategy and a collection of Vβ mutations at the Vβ:SEC3 interface yielded a 14,000 Da protein with 1000-fold increased affinity for SEC3. These small domains may serve as useful antagonists of some T cell mediated diseases, as the engineered protein was effective at inhibiting T cell activation.

The selection and characterization of mutated scTCR genes with stop codons in the linker or the N terminus of the Vα showed that elimination of the Vα region allowed higher expression levels of the Aga-2/Vβ fusion, compared to the Aga-2/scTCR (Vβ-linker-Vα). It is also possible that the presence of Vα might reduce the affinity of the scTCR for SEC3. The Vα region does not directly

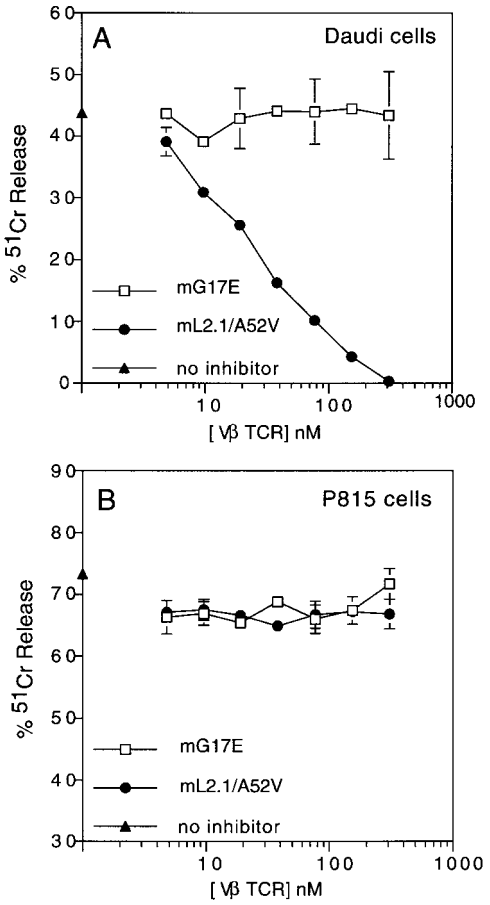

Figure 5. Inhibition of SEC3-mediated cytotoxic T cell activity by soluble, high affinity Vβ mutant mL2.1/A52V. Cytotoxicity assays with CTL clone 2C and ^{51}Cr-labeled target cell lines Daudi (a) and P815 (b) were performed in the presence of various concentrations of the two Vβ proteins, mG17E (open squares) and mL2.1/A52V (filled circles). The class II MHC-positive line Daudi was assayed in the presence of SEC3. P815 expresses the endogenous peptide/MHC (p2Ca/L^d) recognized by clone 2C.

contact SEC3 but a Vα region, and CDR3α in particular, might influence the structure of a Vβ region and thereby affect SEC3 binding indirectly.[15,38] Various earlier studies suggested that Vα regions have an effect on SAg stimulation.[39-41] The strategy used here to isolate the minimal SEC3-binding domain should be applicable to identification and engineering of proteins in general. For example, expression of cDNA libraries and/or gene fragments as Aga-2 fusions would allow one to rapidly select a minimal binding domain using a fluorescent-labeled ligand and flow sorting.

The multiple mutations that were isolated by selection with SEC3 were largely restricted to the Vβ surface that interacts with SEC3. As shown in Figure 4, these mutations were clustered such that it is reasonable to think they act in concert to optimize the interaction energy with SEC3. Gln72, which is within the HV4 region of the Vβ, is located in proximity to the disulfide loop of SEC3 and thus it is possible that the histidine side-chain at this position in the Q72H mutant interacts more effectively with this region. However, the Q72H mutation was selected with SEC3 (round two, see Figure 1) and not with SEC3-1A4. Thus, its contribution to binding, if any, is with wild-type residues of SEC3 or perhaps the backbone of the disulfide loop. Except for residue 72, the mutations identified here appear to interact with regions that are shared between SEC3 and the variant SEC3-1A4. SPR data suggested that the affinity of the mL2.1/A52V Vβ was increased almost 100-fold for SEC3-1A4, consistent with this notion. Affinity measurements based on flow cytometric titrations showed less of an affinity increase for the SEC3-1A4. However, this approach is not a true equilibrium measurement, as a washing step was necessary before final flow cytometric analysis. This could yield affinity values that are qualitative rather than quantitative.

It is interesting to note that valine, selected here in the A52V mutation, is the most common residue present among the non-Vβ8.2, SEC3-reactive Vβ regions (6/13).[12,13] Alanine at position 52 is found only in Vβ8.2. Thus, the *in vitro* selection process described here and the process involved in evolution and selection of human and mouse Vβ regions may have operated in a similar manner. Both Ala52 and Ser54 have multiple contacts with SEC3 in the Vβ8.2-SEC3 complex. This region also represents the binding hot spot, based on alanine scanning mutagenesis results of the Vβ[13] and SEC3[16] proteins. Ala52 contacts SEC3 residue Tyr90 (five vdw contacts) and Ser54 contacts Val91 (one vdw contact[12]). Ser54 also contacts Asn23 in SEC3 (five vdw contacts[16]). Previous studies have shown that an asparagine at position 23 in SEC3 is critical for T cell stimulation[42] and an alanine substitution at this position reduced binding to Vβ8 by 70-fold.[16] In fact, SEC3 residues Tyr90 and Asn23 are strictly conserved in SEC1, SEC2, SEC3, SEB, and SPEA (streptococcal pyrogenic exotoxin A, another SAg that binds Vβ8.2). It is perhaps these interactions that account for the increased binding affinity of the mL2.1/A52V mutant for SEB, a finding that lends some credence to the possibility of isolating a single Vβ mutant that can bind with high affinity to many of these SAgs. As discussed below, Vβ mutants with SAg cross-reactivities could be more effective as antagonists of bacterial infections that have enterotoxin-mediated consequences.

The cluster of mutations near Vβ residues 66 and 80 might also be expected to act cooperatively. For example, the two most significant contact residues in this area of the structure are Lys57 and Lys66. Whereas Lys57 contributed 0.8 kcal/mol of energy to the interaction with SEC3 relative to the TCR with an alanine at this position, Lys66 did not contribute energy to the interaction (i.e. the K66A mutant was actually improved twofold in SEC3

binding, compared to the wild-type[13]). Hence it is understandable that the Vβ region near residue 66 might be a likely candidate for further optimization in the SEC3 interaction. The charge reversal at residue 66 (K66E) and charge neutralization at residue 80 (E80V) may act cooperatively to prevent the ionic repulsion that might occur if two negative charges (mutant K66E and wt E80) were present at these positions (the closest neighboring atoms, K66-C^{ε} and E80-C^{γ} are only 3.37 Å apart). The closest potential charged interaction between E66 in the Vβ mutant and SEC3 is Lys25 of SEC3, which is approximately 5 to 6 Å away. Certainly, structural studies of a mL2.1/A52V-SEC3 complex would provide insight into the mechanisms involved in the overall affinity increase.

The clustering of these mutations is consistent with recent studies of antibody affinity variants. For example, in the 48G7 antibody system several mutations improved the binding affinity only in the context of a specific nearby residue that was also mutated relative to the germline.[43] Thus, separate single-site mutations frequently did not yield the affinity increases that might have been predicted from the double mutant. In the case of anti-(4-hydroxy-3-nitrophenyl) acetyl (NP) antibodies, a $CDR3_H$ residue influenced the affinity maturation pathway achieved through mutations at a $CDR1_H$ residue.[44] Even residues that are not in direct contact with the ligand can have significant longer range effects on binding, as seen in the lysozyme-D1.3 antibody system.[45] Accordingly, we might expect both cooperative effects among mutations in the Vβ mutants and perhaps longer range effects for some of the mutations that are not in direct contact with SEC3. A practical consequence of these observations is that it may be useful to apply mutagenesis methods that are capable of producing variants at adjacent residues in the three-dimensional structure of the protein. Because changes to a single residue can affect the conformation of the protein at distant regions,[35] mutational strategies that co-evolve the entire interface may also be advantageous.

The isolation of a Vβ mutant with nanomolar affinity for SEC3 provided an opportunity to test whether such proteins might be effective antagonists of SAg-mediated reactions. As shown in Figure 5, a soluble form of the highest affinity Vβ mutant, mL2.1/A52V, was a very effective inhibitor of specific T cell activity mediated by SEC3. Consistent with our expectations, the low affinity Vβ8 protein (mG17E/L81S) was unable to block activity. This prediction was based on the calculation that even at the highest concentration tested (300 nM), less than 10% of the SEC3 associated with the target cell would be bound at equilibrium. Thus, it was in fact essential that a higher affinity variant of the TCR be engineered for this purpose. A panel of high affinity Vβ antagonists against many different bacterial SAgs may be useful in the treatment of these diseases, perhaps as an adjunct to therapy with TNFα antagonists. Alternatively, the ability to engineer Vβ mutations at positions that associate with identical residues among different superantigens may allow the generation of a broadly cross-reactive, single antagonist. It would be relatively simple to introduce sequential selections with different superantigens into the yeast display and selection process. A recent report showed that an SEB-derived peptide could act as a superantigen antagonist.[46] However, the molecular basis of its activity was unknown as it was derived from a region that is not involved in either MHC or TCR binding. It is also possible that monoclonal antibodies to SAgs could, in principle, serve as antagonists[47,48] but the engineering of smaller domains (i.e. Vβ regions) that already have evolved to cross-react with different SAGs appear to provide distinct advantages.

Material and Methods

Yeast surface display of 2C scTCR mutants

An *Nhe*I-*Xho*I PCR fragment containing the 2C scTCR (Vβ8.2-linker-Vα3.1 gene[49]) was cloned into the yeast display vector pCT202.[23] Full-length, functional scTCR could only be expressed after selection for mutations that improved stability of the protein and allowed surface display. A mixture of wild-type scTCR and the mutants mTCR7, mTCR15, and mTCR7/15 (containing the stabilizing mutations βG17E and αL43P) was subjected to error-prone PCR in order to produce a new library of scTCR mutants, as described.[26] A library of $\sim 3 \times 10^5$ ligation products was obtained during mutagenesis round one. This process was repeated for mutagenesis round two, except the error-prone PCR library was derived from clone mL1.1, isolated from round one selections. A library of $\sim 2 \times 10^5$ ligation products was obtained during mutagenesis round two. After each round of mutagenesis, mutated plasmids were transformed into EBY100 yeast cells by lithium acetate transformation.[50] The single amino acid Vβ substitutions, A52V and L81S, were introduced using the QuikChange site-directed mutagenesis procedure as described by the manufacturer (Stratagene).

Flow cytometric selection and analysis of mutant scTCR

Expression of scTCR-Aga2p fusions was induced by growth of yeast cells at 20 °C in medium that contained galactose, as described.[23-27] Yeast cell libraries were incubated with limiting concentrations of biotinylated-SEC3-1A4 (round one) or biotinylated-SEC3 (round two; Toxin Technology, Inc.) diluted in phosphate buffered saline/0.1% bovine serum albumin (PBS-BSA). ''Limiting concentration'' was defined as biotinylated-SEC3 at a concentration $\sim 1/10\ K_D$ as determined by flow cytometry equilibrium binding curves.[51]

Cells were incubated with 25 μl of biotinylated-SEC3 on ice for ~45-60 minutes, washed with 0.5 ml PBS-BSA, and pelleted. The pellets were then mixed with 25 μl of a streptavidin-phycoerythrin conjugate diluted 1:200 in PBS-BSA (SAv-PE; PharMingen), incubated on ice for 30 minutes, washed with PBS-BSA, and pelleted. In alternate selection rounds, yeast were double stained with biotinylated-SEC3 and anti-c-*myc* monoclonal antibody 9E10 (1:100 dilution of raw ascites fluid; Berkeley

Antibody Company) followed by a goat anti-mouse $F(ab')_2$ IgG conjugated to FITC (1:50; Kirkegaard and Perry Labs). Immediately before sorting, cells were diluted in an appropriate volume of PBS-BSA. After three cycles of cell sorting on a Coulter 753 bench sorter (Flow Cytometry Facility of the UIUC Biotechnology Center), the collected yeast cells were plated on selective medium to isolate individual clones.

Yeast clones were analyzed by flow cytometry with biotinylated-SEs (SEB, SEC3, or SEC3-1A4), followed by SAv-PE as described above. In some cases, cells were incubated with the anti-c-*myc* antibody 9E10 to estimate surface expression levels of the various mutants. Yeast cells were analyzed on a Coulter Epics XL flow cytometer. Mean fluorescence units (MFU) data obtained in this analysis was plotted as a function of ligand (SE) concentration.

Sequencing of selected TCR mutants

Plasmid DNA from 14 clones from each of the two rounds of mutagenesis (see Figure 1) were recovered and sequenced (W.M. Keck Center for Comparative and Functional Genomics of the UIUC Biotechnology Center). Mutants from the mL1 library contained the following substitutions: mL1.1 (four isolates; βG17E, βS54N, βT87S, βG96V, STOP in linker), mL1.2 (four isolates; βG17E, βS54N, STOP near Vα CDR1), mL1.3 (three isolates; βG17E, βS54N, βA110E, linker K7E, linker D20V, αL43P, αV92 M, αI118T), mL1.4 (one isolate; βG17E, βN24Y, βS54N, βK66R, linker D24H, αQ1R, αS108P, αK111N), mL1.5 (one isolate; βG17E, βS54N, αL43P), and mL1.6 (one isolate; βR9G, βG17E, βS54N, αL43P, αD53G, αW82R). Mutants from the mL2 library contained the following substitutions, in addition to those mutations (βG17E, βS54N, βT87S, βG96V, STOP in linker) found in mL1.1, the template Vβ gene: mL2.1 (eight isolates; βK66E, βQ72H, βE80V, βL81S), mL2.2 (one isolate; βA52V, βL81S), mL2.3 (1 isolate; βA52T), mL2.4 (one isolate; βA52V, c-*myc* KΔE), mL2.5 (one isolate; βA52V), and mL2.6 (one isolate; βA52V, βE73K), mL2.7 (one isolate; βA52V, βG109D).

Expression of soluble Vβ regions

The genes coding for two TCR Vβ mutants (mG17E/L81S and mL2.1/A52V) were subcloned as *Nhe*I-*Bgl*II fragments into plasmid pRS-GAL for soluble expression in yeast as described.[25] The pRS-GAL vector is a centromere-based, low copy plasmid containing a consensus pre-pro sequence and an inducible *GAL 1-10* promoter.[52] The two potential Vβ N-linked glycosylation sites (Asn24 and Asn74) were mutated to glutamine prior to protein expression in yeast in order to reduce potential heterogeneity due to N-linked glycosylation that might effect size filtration during the purification process. It has been shown that glycosylation of Vβ8.2 does not affect TCR binding to SEC3.[15] For expression, Vβ-pRS-GAL constructions were transformed into yeast strain BJ5464 by the lithium acetate method.[50] Yeast from 2 ml of overnight starter SD-CAA cultures (minimal medium containing glucose supplemented with 20 mg/liter tryptophan, trp) grown at 30 °C were transferred to 50 ml of SD-CAA + trp cultures in a 250 ml Erlenmeyer flask. These cultures were grown overnight at 30 °C, transferred to one liter of SD-CAA + trp, and grown for an additional 48 hours at 30 °C. After growth, cells were harvested by centrifugation and the pellet was resuspended in one liter of SG-CAA (minimal medium containing galactose supplemented with trp). These cultures were induced for 72 hours at 20 °C.

Yeast supernatants were harvested by centrifugation, and concentrated to ~50 ml using a stirred cell apparatus (Amicon) fitted with a 3000 molecular mass cutoff cellulose membrane (Millipore). The mutant Vβ proteins (containing a 6-His tag at the C-terminal of the gene product) were purified using Ni-NTA affinity chromatography as described by the manufacturer (Qiagen). Further purification of Vβ protein was performed by HPLC (BioCad Sprint; Perseptive Biosystems, Inc.) using a size exclusion Superdex 200 column (Pharmacia) in PBS (pH 7.3).

Surface plasmon resonance

SPR analysis of mutants mG17E and mL2.1/A52V binding to wild-type SAg SEC3 and the high affinity variant SEC3-1A4 were performed using a BIAcore 1000 instrument (Pharmacia). For the binding experiments, SAgs were immobilized (500 RU each) and various concentrations of mutant Vβ TCR protein were injected for five minutes at a flow rate of 25 μl/minute. The complexes were allowed to dissociate for ten minutes at the same flow rate. The on and off-rates were determined from the association and dissociation curves, corrected for non-specific binding, with their ratio yielding the equilibrium binding constant (K_D). Blank controls in which the TCR proteins were passed over blocked, empty sensor chips were also performed for comparison.

T cell activity assays

^{51}Cr-labeled target cells, MHC class II$^+$ Daudi or P815 peptide-MHC class I$^+$ (peptide-L^d), were added in a volume of 50 μl/well at 10^4 cells/well, in 96-well U-bottom plates. SEC3 (Toxin Technology, Inc.) was added to Daudi cells at a concentration of 25 ng/ml, in a volume of 50 μl/well. Vβ proteins were added at various concentrations to wells containing SEC3/Daudi or P815 target cells. Plates were incubated for 30 minutes at 37 °C, 5 % (v/v) CO_2. Cytotoxic T cells derived from the clone called 2C were added in a volume of 50 μl/well at 10^5 cells/well, after the initial 30 minute incubation. The E:T ratio (effector/target) was 10:1. Plates were centrifuged for five minutes at 800 rpm and incubated an additional four hours at 37 °C. Supernatants (100 μl) were harvested after pelleting the cells by centrifugation and the released ^{51}Cr was measured in a gamma counter. The percentage ^{51}Cr release or target cell lysis was calculated as:

$$\%^{51}\text{Cr release} = \frac{(\text{experimental cpm} - \text{spontaneous release cpm})}{(\text{maximum release cpm} - \text{spontaneous release cpm})} \times 100$$

Data were plotted as % ^{51}Cr release *versus* concentration Vβ inhibitor.

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References

1. Garcia, K. C., Degano, M., Stanfield, R. L., Brunmark, A., Jackson, M. R. & Peterson, P. A. *et al*. (1996). An αβ T cell receptor structure at 2.5 angstrom and its orientation in the TCR-MHC complex. *Science,* **274**, 209-219.
2. Garboczi, D. N., Ghosh, P., Utz, U., Fan, Q. R., Biddison, W. E. & Wiley, D. C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature,* **384**, 131-141.
3. Garcia, K. C., Teyton, L. & Wilson, I. A. (1999). Structural basis of T cell recognition. *Annu. Rev. Immunol.* **17**, 369-397.
4. White, J., Herman, A., Pullen, A., Kubo, R., Kappler, J. & Marrack, P. (1989). The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell,* **56**, 27-35.
5. Marrack, P. & Kappler, J. (1990). The staphylococcal entertoxins and their relatives. *Science,* **248**, 705-711.
6. Bohach, G. A., Fast, D. J., Nelson, R. D. & Schlievert, P. M. (1990). Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit. Rev. Microbiol.* **17**, 251-272.
7. Kotzin, B. L., Leung, D. Y. M., Kappler, J. & Marrack, P. (1993). Superantigens and their potential role in human disease. *Adv. Immunol.* **54**, 99-166.
8. Herman, A., Kappler, J. W., Marrack, P. & Pullen, A. M. (1991). Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* **9**, 745-772.
9. Fields, B. A., Malchiodi, E. L., Li, H., Ysern, X., Stauffacher, C. V. & Schlievert, P. M. *et al*. (1996). Crystal structure of a T-cell receptor β-chain complexed with a superantigen. *Nature,* **384**, 188-192.
10. Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X. & Schlievert, P. M. *et al*. (1998). Three-dimensional structure of the complex between a T cell receptor beta chain and the superantigen staphylococcal enterotoxin B. *Immunity,* **9**, 807-816.
11. Andersen, P. S., Lavoie, P. M., Sekaly, R. P., Churchill, H., Kranz, D. M. & Schlievert, P. M. *et al*. (1999). Role of the T cell receptor alpha chain in stabilizing TCR-superantigen- MHC class II complexes. *Immunity,* **10**, 473-483.
12. Li, H., Llera, A., Malchiodi, E. L. & Mariuzza, R. A. (1999). The structural basis of T cell activation by superantigens. *Annu. Rev. Immunol.* **17**, 435-466.
13. Churchill, H. R., Andersen, P. S., Parke, E. A., Mariuzza, R. A. & Kranz, D. M. (2000). Mapping the energy of superantigen *Staphylococcus* enterotoxin C3 recognition of an alpha/beta T cell receptor using alanine scanning mutagenesis. *J. Exp. Med.* **191**, 835-846.
14. Kappler, J., White, J., Kozono, H., Clements, J. & Marrack, P. (1994). Binding of a soluble αβ T-cell receptor to superantigen/major histocompatibility complex ligands. *Proc. Natl Acad. Sci. USA,* **91**, 8462-8466.
15. Malchiodi, E. L., Eisenstein, E., Fields, B. A., Ohlendorf, D. H., Schlievert, P. M., Karjalainen, K. & Mariuzza, R. A. (1995). Superantigen binding to a T cell receptor β chain of known three-dimensional structure. *J. Exp. Med.* **182**, 1833-1845.
16. Leder, L., Llera, A., Lavoie, P. M., Lebedeva, M. I., Li, H. & Sekaly, R. P. *et al*. (1998). A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor beta chain and major histocompatibility complex class II. *J. Exp. Med.* **187**, 823-833.
17. Eisen, H. N., Sykulev, Y. & Tsomides, T. J. (1996). Antigen-specific T-cell receptors and their reactions with complexes formed by peptides with major histocompatibility complex (MHC) proteins. *Advan. Protein Chem.* **49**, 1-56.
18. Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampl, J., Arden, B. & Chien, Y. (1998). Ligand recognition by alpha beta T cell receptors. *Annu. Rev. Immunol.* **16**, 523-544.
19. Loetscher, H. R., Gentz, R., Zulauf, M., Lustig, A., Tabuchi, H. & Schlaeger, E. J., *et al*. (1991). Recombinant 55 kDa TNF receptor stoichiometry of binding to TNFa and TNFb and inhibition of TNF activity. *J. Biol. Chem.* **266**, 18324-18329.
20. Hebell, T., Ahearn, J. M. & Fearon, D. T. (1991). Suppression of the immune response by a soluble complement receptor of B lymphocytes. *Science,* **254**, 102-105.
21. Winter, G., Griffiths, A. D., Hawkins, R. E. & Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annu. Rev. Immunol.* **12**, 433-455.
22. Weidanz, J. A., Card, K. F., Edwards, A., Perlstein, E. & Wong, H. C. (1998). Display of functional αβ single-chain T-cell receptor molecules on the surface of bacteriophage. *J. Immunol. Methods,* **221**, 59-76.
23. Boder, E. T. & Wittrup, K. D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. *Nature Biotech.* **15**, 553-557.
24. Kieke, M. C., Shusta, E. V., Boder, E. T., Teyton, L., Wittrup, K. D. & Kranz, D. M. (1999). Selection of functional T cell receptor mutants from a yeast surface- display library. *Proc. Natl Acad. Sci. USA,* **96**, 5651-5656.
25. Shusta, E. V., Kieke, M. C., Parke, E., Kranz, D. M. & Wittrup, K. D. (1999). Yeast polypeptide fusion surface display levels predict thermal stability and soluble secretion efficiency. *J. Mol. Biol.* **292**, 949-956.
26. Shusta, E. V., Holler, P. D., Kieke, M. C., Kranz, D. M. & Wittrup, K. D. (2000). Directed evolution of a stable scaffold for T-cell receptor engineering. *Nature Biotechnol.* **18**, 754-759.
27. Holler, P. D., Holman, P. O., Shusta, E. V., O'Herrin, S., Wittrup, K. D. & Kranz, D. M. (2000). *In vitro* evolution of a T cell receptor with high affinity for peptide/MHC. *Proc. Natl Acad. Sci. USA,* **97**, 5387-5392.
28. Udaka, K., Tsomides, T. J. & Eisen, H. N. (1992). A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell,* **69**, 989-998.
29. Tallquist, M. D., Yun, T. J. & Pease, L. R. (1996). A single T cell receptor recognizes structurally distinct

MHC/peptide complexes with high specificity. *J. Exp. Med.* **184**, 1017-1026.
30. Soo, Hoo W. F. & Kranz, D. M. (1993). Role of CD8 in staphylococcal enterotoxin B-mediated lysis by cytotoxic T lymphocytes. *J. Immunol.* **150**, 4331-4337.
31. Schodin, B. A., Tsomides, T. J. & Kranz, D. M. (1996). Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity. *Immunity,* **5**, 137-146.
32. Manning, T. C., Schlueter, C. J., Brodnicki, T. C., Parke, E. A., Speir, J. A. & Garcia, K. C. *et al*. (1998). Alanine scanning mutagenesis of an αβ T cell receptor: mapping the energy of antigen recognition. *Immunity,* **8**, 413-425.
33. Kearse, K. P., Roberts, J. L., Munitz, T. I., Wiest, D. L., Nakayama, T. & Singer, A. (1994). Developmental regulation of alpha beta T cell antigen receptor expression results from differential stability of nascent TCR alpha proteins within the endoplasmic reticulum of immature and mature T cells. *EMBO J.* **13**, 4504-4514.
34. Kearse, K. P., Takahama, Y., Punt, J. A., Sharrow, S. O. & Singer, A. (1995). Early molecular events induced by T cell receptor (TCR) signaling in immature CD4+ CD8+ thymocytes: increased synthesis of TCR-alpha protein is an early response to TCR signaling that compensates for TCR-alpha instability, improves TCR assembly, and parallels other indicators of positive selection. *J. Exp. Med.* **181**, 193-202.
35. Atwell, S., Ultsch, M., De Vos, A. M. & Wells, J. A. (1997). Structural plasticity in a remodeled protein-protein interface. *Science,* **278**, 1125-1128.
36. Boder, E. T., Midelfort, K. S. & Wittrup, K. D. (2000). Directed evolution of antibody fragments with monovalent femtomolar antigen-binding affinity. *Proc. Natl Acad. Sci. USA,* **97**, 10701-10705.
37. Foote, J. & Eisen, H. N. (2000). Breaking the affinity ceiling for antibodies and T cell receptors. *Proc. Natl Acad. Sci. USA,* **97**, 10679-10681.
38. Webb, S. R. & Gascoigne, N. R. (1994). T-cell activation by superantigens. *Curr. Opin. Immunol.* **6**, 467-475.
39. Daly, K., Nguyen, P., Hankley, D., Zhang, W. J., Woodland, D. L. & Blackman, M. A. (1995). Contribution of the TCR α-chain to the differential recognition of bacterial and retroviral superantigens. *J. Immunol.* **155**, 27-34.
40. Liao, L., Marinescu, A., Molano, A., Ciurli, C., Sekaly, R. P. & Fraser, J. D., *et al*. (1996). TCR binding differs for a bacterial superantigen (SEE) and a viral superantigen (Mtv-9). *J. Exp. Med.* **184**, 1471-1482.
41. Bravo de Alba, Y., Marche, P. N., Cazenave, P. A., Cloutier, I., Sekaly, R. P. & Thibodeau, J. (1997). V alpha domain modulates the multiple topologies of mouse T cell receptor V beta20/staphylococcal enterotoxins A and E complexes. *Eur. J. Immunol.* **27**, 92-99.
42. Kappler, J. W., Herman, A., Clements, J. & Marrack, P. (1992). Mutations defining functional regions of the superantigen staphylococcal enterotoxin B. *J. Exp. Med.* **175**, 387-396.
43. Yang, P. L. & Schultz, P. G. (1999). Mutational analysis of the affinity maturation of antibody 48G7. *J. Mol. Biol.* **294**, 1191-1201.
44. Furukawa, K., Akasako-Furukawa, A., Shirai, H., Nakamura, H. & Azuma, T. (1999). Junctional amino acids determine the maturation pathway of an antibody. *Immunity,* **11**, 329-338.
45. Goldman, E. R., Dall'Acqua, W., Braden, B. C. & Mariuzza, R. A. (1997). Analysis of binding interactions in an idiotope-antiidiotope protein- protein complex by double mutant cycles. *Biochemistry,* **36**, 49-56.
46. Arad, G., Levy, R., Hillman, D. & Kaempfer, R. (2000). Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation. *Nature Med.* **6**, 414-421.
47. Hamad, A. R., Herman, A., Marrack, P. & Kappler, J. W. (1994). Monoclonal antibodies defining functional sites on the toxin superantigen staphylococcal enterotoxin B. *J. Exp. Med.* **180**, 615-621.
48. Verdrengh, M., Kum, W., Chow, A. & Tarkowski, A. (1999). The effect of site-specific monoclonal antibodies directed to toxic shock syndrome toxin-1 in experimental Staphylococcus aureus arthritis. *Clin. Exp. Immunol.* **118**, 268-270.
49. Soo, Hoo W. F., Lacy, M. J., Denzin, L. K., Voss, E. W. J., Hardman, K. D. & Kranz, D. M. (1992). Characterization of a single-chain T cell receptor expressed in *E. coli*. *Proc. Natl Acad. Sci. USA,* **89**, 4759-4763.
50. Geitz, R. D., Schiestl, R. H., Willems, A. & Woods, R. A. (1995). Studies on the mechanism of high efficiency transformations of intact yeast cells. *Yeast,* **11**, 355-360.
51. Boder, E. T. & Wittrup, K. D. (1998). Optimal screening of surface-displayed polypeptide libraries. *Biotechnol. Prog.* **14**, 55-62.
52. Shusta, E. V., Raines, R. T., Pluckthun, A. & Wittrup, K. D. (1998). Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nature Biotechnol.* **16**, 773-777.

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