Design of Chimeric Receptor Mimics with Different TcRV\(\beta\) Isoforms

TYPE-SPECIFIC INHIBITION OF SUPERANTIGEN PATHOGENESIS*

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The Staphylococcus aureus enterotoxins (S.E.) A-I, and toxic-shock syndrome toxin TSST-1 act as superantigens to cause overstimulation of the host immune system, leading to the onset of various diseases including food poisoning and toxic shock syndrome. SAgs bind as intact proteins to the DR\(\alpha\)I domain of the MHC class II receptor and the TcRV\(\beta\) domain from the T cell receptor and cause excessive release of cytokines such as IL-2, TNF-\(\alpha\), and IFN-\(\gamma\), and hyperproliferation of T cells. In addition, different SAgs bind and activate different TcRV\(\beta\) isoforms during pathogenesis of human immune cells. These two properties of SAgs prompted us to design several chimeric DR\(\alpha\)l-linker-TcRV\(\beta\) proteins using different TcRV\(\beta\) isoforms to create chimeras that would specifically inhibit the pathogenesis of SAgs against which they were designed. In this study, we compare the design, interaction, and inhibitory properties of three different DR\(\alpha\)l-linker-TcRV\(\beta\) chimeras targeted against three different SAgs, SEB, SEC3, and TSST-1. The inhibitory properties of the chimeras were tested by monitoring IL-2 release and T cell proliferation using a primary human cell model. We demonstrate that the three chimeras specifically inhibit the pathogenesis of their target superantigen. We performed molecular modeling to analyze the structural basis of the type specificity exhibited by different chimeras designed against their target SAgs, examine the role of the linker in determining binding and specificity, and suggest site-specific mutations in the chimera to enhance binding affinity. The fact that our strategy works equally well for SEB and TSST-1, two widely different phylogenetic variants, suggests that the DR\(\alpha\)l-linker-TcRV\(\beta\) chimeras may be developed as a general therapy against a broad spectrum of superantigens released during Staphylococcal infection.

Staphylococcus aureus (S. aureus) bacteria secrete protein exotoxins or superantigens (SAgs)\(^1\) that subvert the host immune system initially through T cell hyperproliferation and massive cytokine release, and eventually cause deletion of the affected T cell population through apoptosis (1–2). The S. aureus SAgs have been implicated as the causative agents in a number of human diseases, including food poisoning and toxic shock syndrome. SAgs have also been shown to promote the onset of chronic conditions such as autoimmune and inflammatory skin diseases by weakening the host immune system (3–5).

The mechanism of S. aureus pathogenesis has been attributed to an alternate mode of SAg binding. In contrast to foreign antigens, SAgs bypass the internalization and processing by the antigen-presenting cell (APC), and instead bind as intact protein externally to the DR\(\alpha\)I domain of the MHC class II receptor on APCs and the V\(\beta\) domain of the T cell receptor (TcR) on T cells (6–9). The alternate binding of SAg to the MHC class II-TcR complex is followed by two additional signaling events between APCs and T cells, the engagement of co-stimulatory ligands and their cognate receptors such as the B7 ligand and CD28 (10) and the involvement of the autocrine and paracrine cytokine network (11). SAgs also have the ability to bind to multiple isoforms of TcRV\(\beta\) (12) and can activate up to 20% of T cells compared with 0.0001% by a conventional antigen, resulting in the activation of a large population of T cells (13).

Until a decade ago, staphylococcal infection was effectively treated with the antibiotics, methillicin (14) and vancomycin (15). However, the emergence of antibiotic-resistant S. aureus has prompted development of several non-antibiotic-based therapies (16), including superantigen-based vaccines (17), small peptide inhibitors of SAg gene production (18), and small SAg-derived neutralizing peptides (19). We have taken a structure-based (or rational design) approach to construct bi-specific chimeric inhibitors composed of the DR\(\alpha\)I domain of MHC class II and V\(\beta\) domain of the TcR connected by a flexible (GSTAPPA)\(_2\) linker. We have based our protein design on the crystal structures of SAgs (20–22) and their complexes with the MHC class II and T cell receptors (6–9). Based on the structure-function relationship between SAgs and their receptors, we expect these chimeras to bind competitively and prevent the SAg from binding to the MHC class II receptor of APCs and the TcR of T cells. Given that different SAgs exhibit different patterns of TcRV\(\beta\) usage (12), it is possible to choose a TcRV\(\beta\) isoform that is specific to a given SAg. Thus, the use of different TcRV\(\beta\) isoforms in our chimeras may impart specificity toward a particular SAg but not to others.

Using this strategy, we have previously reported that a DR\(\alpha\)l-(GSTAPPA)\(_2\)-TcRV\(\beta\) chimera inhibited SEB-induced IL-2 cytokine release and T cell proliferation in a mixture of human peripheral mononuclear and dendritic cells (23). The V\(\beta\) isoform has been shown to be activated by S. aureus SEB (12). Our approach, which employs the use of a chimeric protein to block the initial step of SAg pathogenesis, namely the ligation of APC and T cells, has several unique features. Although the individual DR\(\alpha\)l and TcRV\(\beta\) domains of the chimera show poor binding to their target sites on the SAg, the SAg-specific chimera bound to SEB with micromolar affinity, which is com-

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\(^1\)The abbreviations used are: SAg, superantigen; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; RMS, root mean-squared; TcR, T cell receptor; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cells; DC, dendritic cells.
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Cloning of Chimera Genes—Construction of the SEB chimera has been previously described (23). For the TSST-1 and SEC3 chimeras, the DRα1 and linker domain was amplified from the SEB construct using the primers 5'-gatagcatactgataaagagaaagtg-3' and 5'-gatagctagagctgtccgccgtg-3' to add BglII (bold) and Nhel (underlined) sites to the 5' - and 3'-ends, respectively. The DRα/linker region was ligated into BamHI/Nhel-digested pRSETc vector (Stratagene) using T4 ligase (NEB). The pRSETc vector introduces an N-terminal His6 tag that allows protein purification with the TALON metal affinity resin (Clontech), and purification of the His-tagged chimeras was performed according to the manufacturer's instructions. Briefly, the lysates were rocked for 20 min at room temperature, the resin washed twice with 20 mM Tris-Cl, 100 mM NaCl, 5 µM urea, 1% Tween-20 (pH 8.0), homogenized using a probe sonicator, and centrifuged at 50,000 rpm for 30 min. The cell pellets were resuspended in 20 mM Tris-Cl, 100 mM NaCl, 5 µM urea, 1% Tween-20, 10 mM imidazole (pH 5.3). The proteins were dialyzed stepwise in decreasing concentrations of urea: 1% PBS, 1% Tween-20, 40 mM urea; 2% PBS, 1% Tween-20, 20 mM urea, and finally 3% PBS, 1% Tween-20. Following quantitation using BCA protein assay reagents (Pierce), proteins were stored at 4 °C and used in experiments within 2 weeks.

Human Cell Culture and Inhibition Studies—The SEB, TSST-1, and SEC3 toxins were purchased from Toxin Technology, Inc. Human donor-matched peripheral blood mononuclear cells (PBMCs) and dendritic cells (DCs) (Clonetics) in a 20:1 ratio were cultured in LGM3 media supplemented with 10% donor matched human serum at 37 °C in 5% CO2. For dose response curves, increasing concentrations of either SEB or TSST-1 were incubated with ~10^6 PBMCs/DCs in 1 ml for 10 h. In the inhibition studies, either 20 µl SEC3 or 50 µl TSST-1 were incubated with increasing concentrations of the three chimeras, 5×, 10×, or 20× the molar concentration of the SAgs or left untreated for 1 h at 37 °C. The SAgs/chimera mixtures were then incubated with ~10^6 PBMCs/DCs for 10 h at 37 °C.

Cell supernatants were collected and IL-2 release measured using the standard sandwich ELISA. 2 µg/ml of capture IL-2 antibody (BD Pharmingen) were adhered to Nunc Immunosorp 96-well plates overnight in 0.1% sodium carbonate, pH 9.5. The following day, wells were washed with phosphate-buffered saline (PBS) twice, blocked with 5% BSA for 1 h, and incubated with cell supernatants for 2 h. Wells were then incubated with 1 µg/ml of biotinylated detection anti-IL-2 antibody (BD Pharmingen) for 1 h. After washing, followed by 1:1000 dilution of streptavidin-HRP, for 1 h each. Signal was developed using the Substrate Kit (BD Pharmingen) and read on a Multiskan Plus plate reader (Fisher Scientific, Springfield, NJ), and concentrations of IL-2 were determined against a standard curve using defined amounts of recombinant IL-2 in ELISAs performed in parallel.

Cell Proliferation—T-cell proliferation was measured using the ViaLight HS cell proliferation/cytotoxicity kit (BioWhittaker) according to the manufacturer’s instructions. Briefly, 10^5 PBMCs/DCs (20:1) were plated into flat-bottom, 96-well tissue culture plates in 200 µl of complete RPMI media and treated with 1 µg/ml TSST-1 or 1 µg SEC3 in the presence or absence of varying concentrations of the purified chimeras for 4 days. Only 10^5 PBMCs were used for stimulation with 1 µg SEB. 10 µl of Nucleotide Releasing Reagent was added to each well for 15 min to extract the ATP, followed by 20 µl of ATP Monitoring Reagent. The plate was then loaded into a luminometer (Turner Designs, Inc.) within 10 min of addition of the ATP Monitoring Reagent for measurement of light emission.

Molecular Modeling—The molecular modeling consisted of three steps.

1. The SAgs and their binding partners, MHC class II receptor and TcRV, do not exhibit any significant structural changes upon complex formation. Also, structural studies indicate that SEB, SEC3, and TSST-1 engage in a similar spatial arrangement during contact with the MHC class II receptor (13, 28). Thus, the initial models of the SAg/chimera complexes were constructed based on the crystallographic data as well as biochemical information on intermolecular contacts proposed in the two models. The SAgs were used to identify structural alignments with the Protein Data Bank (PDB): 1) 1SEB, 1SBB, for the SEB/chimera complex; 2) 1JKJ and 1SEB for the SEC3-chimera complex; and 3) 1SEB, 2TSS, and 1YFT for the TSST-1-chimera complex. The DRA1 model for all three chimeras was constructed from the single crystal structure of SEB-MHC class II (1SEB). BLAST (28) searches were performed with the PDB enabled to identify structural alignments with the highest sequence identity (88–92%) with the three different TcRV β chains (Vβ8, Vβ2, and modified Vβ8.2) used in the three chimeras. We used homology modeling to construct the initial models of the TcRV β domains of the chimeras (30).

Protein Overexpression and Purification—The pRSETc vectors containing each of the three chimeras were transformed into BL21 E.coli competent cells. Cells were induced for expression with 2 mM IPTG for 5 h, lysed with 1 mg/ml lysozyme for 15 min, followed by treatment with 10 µg/ml DNase and 10 µg/ml MgCl2 for an additional 30 min, and centrifuged at 50,000 rpm for 30 min. The cell pellets were resuspended in 20 mM Tris-Cl, 100 mM NaCl, 5 µM urea, 1% Tween-20 (pH 8.0), homogenized using a probe sonicator, and centrifuged at 50,000 rpm for 30 min. The resultant supernatants were incubated with TALON Metal Affinity Resin (Clontech), and purification of the His-tagged chimeras was performed according to the manufacturer's instructions. Briefly, the lysates were rocked for 20 min at room temperature, the resin washed twice with 20 mM Tris-Cl, 100 mM NaCl, 5 µM urea, 1% Tween-20, 10 mM imidazole (pH 5.3). The proteins were dialyzed stepwise in decreasing concentrations of urea: 1% PBS, 1% Tween-20, 40 mM urea; 2% PBS, 1% Tween-20, 20 mM urea, and finally 3% PBS, 1% Tween-20. Following quantitation using BCA protein assay reagents (Pierce), proteins were stored at 4 °C and used in experiments within 2 weeks.

Modeling of the DRα1 and linker domain. This amplified the cloning DRA1/linker/TcRVβ sequence using the primers 5'-gatagcatactgataaagagaaagtg-3' and 5'-gagagactataaggctgacgtg-3' to add BglII (bold) and Nhel (underlined) sites to the 5' - and 3'-ends, respectively. The DRA1/linker region was ligated into BamHI/Nhel-digested pRSETc vector (Stratagene) using T4 ligase (NEB). The pRSETc vector introduces an N-terminal His6 tag that allows protein purification with the TALON metal affinity resin (Clontech).
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The relative spatial positions of DR1 and TcRVβ with respect to the SAggs were derived from the three crystal structures: bipartite complexes (SEB-MHC class II: 1SEB), (SEB-TcR: 1SBB), and (SEC3-TcR: 1JCK) by superposition of the complexes. In the case of the TSST-1 complex, conserved structural features of the toxins such as the α-helices α1 and α2 (Figs. 4 and 5) were chosen to orient the SAg in the complex. The relative positions of DRα1 and TcRVβ with respect to TSST-1 were fixed based on structural studies (7, 31). The linker (GSTAPPA), was placed between the C terminus of DRα1 and the N terminus of TcRVβ. Each SAg-chimera complex was minimized locally in vacuum without any constraints using the AMBER 4.1 force field (32); the parm94 parameter set was applied with a dielectric constant of 80 to a gradient limit <10–2 kcal/mol Å⁻². Molecular dynamics simulations of the three chimera-SAg complexes were performed at 300K for 200 ps in vacuum with a dielectric constant of 80 with the energy-minimized model as the t = 0 configuration. Both, intra- and intermolecular constraints were imposed in the simulation. All intra- and intermolecular constraints were based on the available crystal structures (1SEB, 1SBB, 1JCK, 2TSS, 1FYT). Intramolecular constraints (force constants were set to 5 kcal/mol Å⁻²) were used to maintain the native folds of the SAggs, DRα1, and TcRVβ. The “weaker” intramolecular constraints (force constants set to 2 kcal/mol Å⁻²) were applied to allow movement and sufficient flexibility of the partners in the complex. Intermolecular contacts for the SEB-chimera complex were derived from the following crystal structures SEB-DRα1: 1SEB and SEB-TcRVβ: 1SBB. Intermolecular contacts for the SEC3-chimera complex were adopted from the crystal structure of SEC3-TcRVβ complex (1JCK) and the observed interactions in the homologous crystal structure of DRα1–SEC2 complex (27). In the case of the TSST-1-chimera, experimental contact information is available only for the TSST-1-DRα1 interaction (7). No intermolecular constraints were employed between TSST-1 and TcRVβ. No constraints were applied to the (GSTAPPA) linker. After an equilibration period (~100 ps), 100 snapshots (one after every picosecond) were isolated for the 101–200 ps segment of the MD trajectory and minimized. An average structure of the 100 minimized snapshots was also calculated and minimized.

In a systematic analysis of the 100 minimized snapshots and the minimized average structure revealed the nature of various pair-wise interactions and their variations around the average values. A root mean-square (RMS) matrix was computed for the 100 minimized snapshots to determine the conformational variations among the low energy structures of each SAg-chimera complex. RMS deviations were computed between the minimized average structure and each of the 100 minimized snapshots to identify snapshots that were most closely or distantly related to the minimized average structure of the complex. To define the pairwise contacts between SAg and specific chimera, we calculated changes in solvent accessibility for each residue using the program MOLMOL (33) in the free SAg and the free chimera as well as in the SAg-chimera complex. A threshold of 3% change in the solvent accessible surface per residue was used to define a contact between the free SAg and the chimera due to complex formation. Thus, all residues that show a change in solvent accessible surface larger than 5% upon complex formation are defined as contact residues.

RESULTS

Design of SAg Type-specific Chimeric Inhibitors—We have previously reported the design of a chimeric protein targeted against SEB (SEBc). This chimera, composed of the DRα1 domain of the MHC class II receptor linked to the Vβ3 domain of the T cell receptor, inhibited IL-2 release and T cell proliferation in a 20:1 mixture of PMBCs and DCs (23). The appropriate length and sequence of the DRα1 and TcRVβ domains were chosen to retain the native structural folds present in the full-length MHC class II and T cell receptors. Two invariant cysteines form a disulfide bond in the native fold as well as the contacting CDR1, CDR2, and HV4 loops were kept in the TcRVβ sequences. The DRα1 and TcRVβ domains were linked by two copies of GSTAPPA, a linker sequence derived from the human tandem repeat protein mucin and shown to be flexible and non-antigenic (34). The SEB/SEBc complex exhibited a Kd ~6 μM, which is in the range of binding affinities (Kd ~1–100 μM) measured between SAggs and their natural binding partners, the MHC class II and T cell receptors (24–26).

Using the same strategy for structure-based design, we have constructed two additional chimeric inhibitors targeted against TSST-1 and SEC3. Both consist of the same DRα1 and linker sequence as the chimera targeted against SEB, but fused to different TcRVβ isoforms (Fig. 1). For the TSST-1 chimera (TSST-1c), we have chosen the TcRVβ2 isoform, which has been shown to be specifically stimulated by TSST-1 (12). TSST-1 is the most distantly related (~28% amino acid similarity) of all the S. aureus SAggs and represents a distinctly different target for chimera development compared with the more closely related SEB and SEC3. For the SEC3 chimera (SEC3c), we used a truncated, mutagenized form of the murine Vβ8.2 isoform, mL2.1A52V, which had been affinity-enhanced for binding to SEC3 and displayed a >1000 fold increase in binding to SEC3, Kd ~7 nm (27). We chose this mutagenized Vβ8.2 isoform for the construction of SEC3c to try and obtain a chimera with enhanced inhibitory properties.

Type-specific Inhibition of IL-2 Release by Chimeras—The three chimeric inhibitors, SEBc, TSST-1c, and SEC3c, were expressed in E. coli and purified using TALON metal affinity resin that binds to the His tag at the N termini of the proteins. We performed ELISAs to measure SAg-induced IL-2 release in the presence of each of the chimeric proteins to determine whether the different chimeras specifically inhibited the cytokine release induced by the SAg against which the chimera was designed. To determine the SAg concentration to use for cell activation and incubation with the inhibitory chimeras, donor-matched PBMCs, and DCs in a 20:1 ratio were stimulated with increasing concentrations of TSST-1, SEC3, or SEB to measure the dose response. Optimally, inhibition tests for the chimera should be conducted with stimulated cells that release intermediate levels of cytokine release (~40–100 pg/ml at 10 h). Any inhibitory effects mediated by the chimera may be difficult to observe in barely-stimulated (IL-2 release <10 pg/ml at 10 h) or overstimulated (IL-2 release ~500 pg/ml at 10 h) cells. Concentrations of 50 pg TSST-1 and 20 pg SEB stimulated ~68 and ~47 pg/ml IL-2 release, respectively (data not shown), and these concentrations were used in the inhibition of cytokine release assays. In the case of SEB activation, the PMBCs/DCs from several different donors exhibited more pronounced sensitivity, with sub-pg SEB concentrations stimulating >500 pg/ml IL-2 release (data not shown). Given that the use of such small quantities of SEB toxin resulted in such substantial levels of cytokine release, we focused on TSST-1 and SEC3-stimulated PMBCs/DCs for our inhibition studies.

TSST-1 or SEC3 was incubated with 5x, 10x, and 20x the SAg concentration of each of the three purified chimeras, SEBc, TSST-1c, and SEC3c. The resultant chimera/SAg mixtures were subsequently incubated with 10⁶ (20:1) PMBCs/DCs for 10 h to stimulate cell activation. Cell supernatants were then collected, and IL-2 release was measured by ELISA. The TSST-1 and SEC3 chimeras inhibited IL-2 release in a SAg type-specific and concentration-dependent manner, when compared with cells stimulated with SAg alone (Fig. 2, A and B). TSST-1c at 10x and 20x concentration showed ~25 and ~55% reduction in IL-2 release, respectively. In contrast, SEBc and SEC3c appeared to have minimal effects in inhibiting IL-2 release in TSST-1-stimulated cells. Similarly, SEC3c specifically inhibited IL-2 release in SEC3-stimulated cells by ~45 and ~80% at 10x and 20x concentrations of SEC3c, respectively, whereas TSST-1c and SEBc did not exhibit any significant inhibition. Given that the DRα1 and linker domains in the three chimeras are identical, these results indicate that the use of different TcRVβ isoforms imparted specificity to the chimeric proteins in the inhibition of SAg-stimulated cells.

Type-specific Inhibition of Cell Proliferation by Chimeras—In addition to measuring cytokine release, we also assessed...
whether incubation of SAgs with the different chimeras inhibited T cell proliferation in a SAg type-specific manner. We determined the appropriate SAg concentrations to stimulate our PBMC/DC model system by obtaining dose response curves using the Viability cargo proliferation assay (data not shown). In this method, cell proliferation is measured by bioluminescent detection of cellular ATP content, which is increased in proliferating cells. As we had observed for cytokine release, SEB was significantly more potent in stimulating T cell proliferation. 1 pm SEB was sufficient to stimulate T cell proliferation in PBMCs alone, without the DCs, to a level comparable to that induced by 1 nm TSST-1 and SEC3 in the PBMC/DC mixture.

PBMCs/DCs (20:1) were treated with either 1 nm TSST-1 or 1 nm SEC3 and PBMCs alone were treated with 1 pm SEB in the absence or presence of increasing molar concentrations of the purified chimeras for 4 days. In cells treated with SEB, 20× concentration of SEBc inhibited cell proliferation by ~30% (Fig. 3). In comparison, at 20× concentration TSST-1c had a minimal effect on T cell proliferation in SEB-treated cells. Stimulation with 1 nm TSST-1 or SEC3 exhibited a similar type-specific chimera inhibition of T cell proliferation. At 20× concentration, TSST-1c and SEC3c inhibited T cell proliferation by ~40% in TSST-1-stimulated cells and ~50% in SEC3-stimulated cells, respectively. SEBc did not significantly inhibit cell proliferation in either TSST-1 or SEC3 treated cells. These results are consistent with the cytokine release data in which a chimeric protein can inhibit the cellular effects induced by its corresponding SAg against which the chimera was designed. These type-specific interactions indicate that different TcRVβ sequences can be used to as specific ligands to differentiate between the various SAgs.

Analyses of the SAg-Chimera Complexes by Molecular Modeling—To examine the structural basis for the type-specific behavior, we performed molecular modeling and compared the interactions between SEB, TSST-1, and SEC3 and their specific DRA1/Linker/TcRVβ chimeras. For each of the three complexes, the RMS deviations for the backbone atoms were computed to be within 3.6 Å among the 100 minimized snapshots from the MD trajectory. Also for each complex the RMS deviations for the backbone atoms were computed between the minimized average structure and each of the 100 minimized snapshots. This value was always below 2.5 Å. These relatively low RMS deviations demonstrate that the minimized average structure is a representative model of the ensemble. Fig. 4, A–C show the structural models of the three complexes, SEB/SEBc, SEC3/SEC3c, and TSST-1/TSST-1c.

The DRA1 domain in the three chimeras forms a α/β fold with four anti-parallel β-strands, and one short and one long α-helix. The TcRVβ domain consists of an immunoglobulin-like β-fold containing seven β-strands that flank the complementarity determining regions CDR1, CDR2, and hypervariable loop HV4 (Fig. 1). The major contact areas of the DRA1 domain (cyan, loop 1, loop 2, and the long α-helix) of SEC3c show a more compact interface with SEC3c compared with the SEB complex. Crystallographic studies also suggest a slightly different binding mode of SEC3c to the MHC class II receptor compared with SEB (35).

MD simulations preserved all the major contact areas between the chimeras and their target SAgs. Fig. 5 depicts the residues of the SAgs (underlined) that have been reported to be in contact with DRA1 and TcRVβ. However, we also observe
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Results shown are an average of at least three independent experiments.

PBMCs/DCs were incubated with 1 nM SEC3 either alone or in the presence of increasing molar concentrations of SEBc, TSST-1c, or SEC3c for 10 h. Supernatants were collected and assayed for IL-2 release by ELISA.

PBMCs/DCs were incubated with 1 nM SEC3 either alone or in the presence of increasing molar concentrations of SEBc, TSST-1c, or SEC3c for 4 days. Cell proliferation was then assayed using the Vialight assay to measure cellular ATP. Results shown are an average of at least two independent experiments.

Analysis of Pairwise Contacts between SAgs and Their Respective Chimeras—A detailed analysis of the pairwise contacts between the SAgs and the specific chimeras reveals both similarities and differences in their specific binding behavior (Fig. 5). In general, SEB and SEC3 contact their respective TcRVβ domains in a similar manner, whereas TSST-1 interacts with its Vβ domain in a distinctly different manner (Fig. 4). Unique contacts between TSST-1 and TcRVβ are maintained at β3 and the central helix α3 (Fig. 5). Interactions between SEB or SEC3 and their respective Vβs are present in the loop region between β11/α1, which are not present in TSST-1. In addition, simultaneous DRA1 and TcRVβ contacts were only detected for SEC3 (residues 209 and 210), but not for the corresponding residues in SEB. However, there are similarities for the TcRVβ contacts for all three SAgs, primarily in the region of α1, and at the loops between β2/β3 and α3/β10. TSST-1 contacts with Vβ in α1 are further extended into β1. In the loop region between β2 and β3, the specificity of contacts toward TcRVβ varies, in that SEB forms contacts with Vβ only, while SEC3 and TSST-1 maintain multiple Vβ/linker contacts. Although all three SAgs maintain Vβ contacts in the loop region between α3/β10, the SAgs contact different binding sites in the Vβ domain (SEB-CDR2, SEC3-HV4, and TSST-1-CDR1).

All SAgs show DRA1 contacts in the loop region between β1 and β2, as well as in the region of β3 and α2 (Fig. 5). These contacts are maintained for the different SAgs by all three contact areas of the DRA1 domain, loop1, loop2 and the long α-helix. In TSST-1, the β3/α2 region (residues 43–58) shows a greater flexibility than that in SEB and SEC3. Thus, the contacts in TSST-1 are extended compared with SEB and SEC3 and include Thr-57 and Lys-58. Differences in the DRA1 contacts are mainly located in the flexible loop of α4 regions. Both SEB and SEC3 show contacts involving these regions. SEC3 maintains a slightly different relative orientation toward
DRα1 compared with SEB, thus forming additional contacts that extend from α4 toward β11. For TSST-1, the α4 contacts are missing, while the flexible loop region interacts with the TcRVβ and linker regions rather than DRα1. In addition, a tyrosine/DRα1 contact at residue 115 of TcRV is only observed for SEB and SEC3. This contact stabilizes the SEB/SEC3 specific salt bridge between SAg residue Glu-67 and DRα1 residue Lys-39. This specific salt bridge as well as the stabilizing tyrosine contact is missing in TSST-1. In addition, we detect several hydrophobic interactions, including contacts between SEB/
SEC3 (Phe-44, Tyr-94) and DRα1 L60, and TSST-1 Leu-30, Ile-46, and DRα1 Ile-63, Ala-61, respectively.

The contact region between β4 and β5, which comprises the flexible loop region (Fig. 5, black underlined), contains multiple contacts in all SAg models, and contacts in this area differ the most. All possible combinations of multiple contacts exist for SEB and SEC3 due to the close proximity of the contact epitopes of TcRVβ and DRα1 in this region and the flexibility of the linker. For TSST-1, the loop region contacts only the linker and TcRVβ domains, not the DRα1 domain. The loop region had been previously reported to contact the DRα1 and β chains, as well as the antigenic peptide (7). Possibly, the dynamic nature of the flexible loop region, common to all SAgS, may cause this difference.

Finally, our modeling studies indicate that the (GSTAPPA)_2 linker can support simultaneous binding of the DRα1 and TcRVβ domains to all three SAgS, even though the spatial arrangement of the binding epitopes on the SAg surfaces are very different. Contacts to the linker were detected in the flexible loop region for all three SAgS and in the loop region between β2 and β3 for SEC3 and TSST-1. Differences in linker interactions (number of contact residues) are most likely due to the different spatial positions of the DRα1 and TcRVβ contact epitopes on the SEB/SEC3 and TSST-1 surface.

**DISCUSSION**

Here we describe the design of two additional chimeras, namely SEC3c and TSST-1c, and compare the inhibitory properties of SEBc, SEC3c, and TSST-1c on an experimental and structural basis. We demonstrate that the three chimeric receptor mimics, SEBc, SEC3c, and TSST-1c, inhibit cytokine release and T cell proliferation in a type-specific manner. Since the TcRVβ and DRα1 contact epitopes in the chimeras consist of non-contiguous amino acids (Fig. 5), it was essential to maintain the native folds of the TcRVβ and DRα1 to produce effective inhibitors. The inhibitory activity exhibited by the three receptor mimics indicate that the native DRα1 and TcRVβ folds are maintained, and that the TcRVβ domains do, indeed, impart SAg specificity to the chimeras. The chimeras are able to distinguish not only two distantly related SAg family members, SEB and TSST-1, but also the more closely related SEB and SEC3 (36).

The observation that the DRα1-linker-TcRVβ chimeras are effective against several SAgS is significant in terms of their practical use against *S. aureus* infections. During the onset of *S. aureus*-based diseases such as food poisoning or systemic shock, multiple superantigens are released in the body to cause productive infection. While antibiotics can potentially kill the whole bacterium and stop the release of all superantigens, the emergence of antibiotic-resistant *S. aureus* strains necessitates the development of alternative measures to protect against the released superantigens, the primary causative agents of infection. Therefore, our therapeutic approach, based upon the concept of using rationally designed receptor mimics that can be effective inhibitors against multiple superantigens, may prove to be a viable strategy against antibiotic-resistant *S. aureus*. Our finding that the DRα1-linker-TcRVβ receptor mimics are effective against different SAgS is a strong validation of our hypothesis.

From our molecular modeling studies, we show that the three SAgS contact the DRα1 domain in a similar manner, although there are slight differences in the relative orientation between SAg and DRα1. The major differences in specificity occur at the contact regions between the Vβ domains and SAgS, as expected from our original design of using different TcRVβ isoforms to impart specificity to our chimeras. That SEB/SEBc and SEC3/SEC3c complexes share similar contact residue profiles but differ from TSST-1/TSST-1-c is also not surprising, given that TSST-1 shares only ~28% homology with other SAg family members, compared with ~60% homology between SEB and SEC3 (37). However, as discussed above, there are also observable differences in the SEB/SEBc and SEC3/SEC3c complexes. These differences include the loop region between α3/β10, in which Phe-177 in SEB contacts CDR2 of the Vβ3 domain, whereas Phe-176 and Asn-177 of SEC3 contacts HV4 of the Vβ8.2 domain. In addition, only SEC3 maintains simultaneous DRα1 and Vβ contacts at D209/Q210 and interactions with the linker in the loop region between β2/β3. These differences most likely contribute to the ability of SEBc and SEC3c to specifically inhibit their respective chimeras despite the relatively high level of homology between SEB and SEC3.

Because SEC3c exhibited the highest relative level of type-specific inhibition in both IL-2 release and T cell proliferation compared with the other chimeras, it was of interest to examine whether the observed inhibition was due to the specific choice of Vβ3 isoform in the SEC3c. As previously mentioned, we chose a truncated version of the mouse Vβ8.2 isoform originally
obtained by screening a mutagenized library of Vβ8.2 variants using yeast display and flow cytometry sorting (27). This Vβ8.2 variant displayed a Kd of 7 nM for binding to SEC3, which is 1000 fold lower than for the original Vβ8.2 isoform. The mutagenized Vβ8.2 isoform in SEC3c was truncated and contains seven out of the nine mutations in the original mL2.1/A52V mutant (see Fig. 1), since the additional two mutations lay outside our consensus Vβ domain used for SEBc and TSST-1c (27). While the truncated Vβ8.2 isoform in SEC3c may not display exactly the same binding properties of its full-length counterpart, we examined how the seven mutations changed the residue contact profile with SEC3 using molecular modeling. Indeed, the calculated relative interaction energy for the native Vβ8.2 sequence is higher (−3.5 kcal/mol) upon complex formation compared with the mutated, truncated Vβ8.2 sequence, indicating that the Vβ8.2 sequence in SEC3c yields a more stable complex and most likely enhanced the inhibitory properties of SEC3c.

The DRα1-linker-TrcRVβ chimeras were designed to competitively bind to the SAgs, thus blocking the initial step of SAg pathogenesis and downstream activation of immune cells. Although we have demonstrated the type-specific inhibitory properties of our chimeras in cell culture studies, affinity enhancement of the chimeras to increase binding affinity for the different SAgs would produce a more effective inhibitor, especially for the practical implementation of the chimeras in therapy. Production of chimeras with a much higher binding affinity to SAg compared with the Kd(μM) displayed by SAg binding to the MHC class II and T cell receptors (24–26) would more effectively prevent SAgs from binding their receptor targets and forming an immune synapse to activate immune cells.

Affinity enhancement can be achieved either by in vitro evolution or by site-directed mutagenesis based on our molecular modeling studies. Since the TrcRVβ domain imparts SAg binding specificity, affinity enhancement of the TrcRVβ domain may yield receptor mimics with more enhanced inhibitory properties against a given SAg. The mutagenesis of the Vβ8.2 isoform to increase binding affinity for SEC3 by 1000 fold (27) strongly supports this strategy as being particularly effective. Currently, we have successfully expressed SEBc on the yeast cell surface to confirm that yeast display can be used with our chimeric scaffold, and we will proceed to the selection of high affinity binders to SAgs in our future studies. Use of the yeast display system may also lead to selection of TrcRVβ variants that are cross-reactive and bind to several SAgs with high affinity. Possibly, such a TrcRVβ variant in our scaffold would produce a single cross-reactive chimera that is effective in the treatment of a S. aureus infection in which several different SAgs are released.

In addition to random mutagenesis, we can also introduce site-specific mutation that were identified from our modeling studies to increase the stability of the interaction complex between SAgs and their specific chimeras.2 Interestingly back-
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