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Neutralization of staphylococcal enterotoxin B by soluble, high-affinity receptor antagonists

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Exotoxins of *Staphylococcus aureus* belong to a family of bacterial proteins that act as superantigens by activating a large subset of the T-cell population, causing massive release of inflammatory cytokines. This cascade can ultimately result in toxic shock syndrome and death. Therapeutics targeting the early stage of the pathogenic process, when the superantigen binds to its receptor, could limit the severity of disease. We engineered picomolar binding affinity agents to neutralize the potent toxin staphylococcal enterotoxin B (SEB). A single immunoglobulin-like domain of the T-cell receptor (variable region, Vβ) was subjected to multiple rounds of directed evolution using yeast display. Soluble forms of the engineered Vβ proteins produced in *Escherichia coli* were effective inhibitors of SEB-mediated T-cell activation and completely neutralized the lethal activity of SEB in animal models. These Vβ proteins represent an easily produced potential treatment for diseases mediated by bacterial superantigens.

Produced primarily by *Staphylococcus aureus* and *Streptococcus pyogenes*, superantigens (SAgs) are a large family of secreted proteins that act by stimulating cells of the immune system[1,2]. SAgs act by binding to Vβ regions of the T-cell receptor (TCR) and to class II MHC molecules, thereby activating T cells and initiating a systemic release of inflammatory cytokines. This results in a condition known as toxic shock syndrome (TSS), characterized by high fever, erythematous rash and hypotension, which can lead to multiorgan failure and death. The SAg staphylococcal enterotoxin B (SEB) is considered to be a potential biological weapon owing to its toxicity and to previous biowarfare programs[3]. Together with toxic shock syndrome toxin-1 (TSST-1) and SEC1-3, SEB is considered to be a primary causative agent of staphylococcal TSS (refs. 4–6). The structural basis of SAg action has been elucidated over the past 10 years[7]. Although the affinity of SEB for Vβ8 is quite low (K_d = 144 μM)[7], the stimulatory activity of this toxin is very high: incapacitating or lethal doses are estimated to be in the range of nanograms per kilogram body weight[3]. In previous studies, we explored possible therapeutics for SAg-mediated diseases by increasing the affinity of the Vβ domain, allowing for its use in soluble form as a potential neutralizing agent at low toxin concentrations[8,9]. Here we describe the engineering of a TCR Vβ region to generate picomolar-affinity receptor antagonists of SEB. We found that soluble Vβ proteins blocked SEB-mediated activity *in vitro* and completely neutralized the toxin in rabbit models, even in rabbits that had already developed early signs of TSS.

To engineer high-affinity receptor antagonists for SEB, we cloned the mouse Vβ8.2 domain into the yeast display vector pCT202 (ref. 10 and **Fig. 1a**). For affinity maturation of Vβ8, we generated five successive libraries (G1 through G5) containing various site-directed or random mutations within the SEB:Vβ8 interface (**Fig. 1b**); high-affinity clones from each library were selected with biotinylated SEB and high-speed flow sorting (**Supplementary Note** and **Supplementary Table 1** online). Most clones from each library were positive for binding to SEB, whereas wild-type Vβ was undetectable at all concentrations tested (**Fig. 1c** and **Supplementary Fig. 1** online). We analyzed sequences from each generation in order to determine the location of the mutations, and to evaluate their possible mechanisms of action (**Fig. 1d** and **Supplementary Note**).

To further characterize Vβ8 mutants and to examine their effectiveness as neutralizing agents, several clones were expressed in *E. coli* and refolded from inclusion bodies. We measured the binding affinity and kinetics of their interactions with SEB using surface plasmon resonance (SPR) (**Supplementary Table 2** online). Each successive generation of Vβ clones exhibited increased affinity for SEB (**Supplementary Figs. 2** and **3** online): for example, second-generation clone G2-5 bound SEB with an affinity of 650 pM (**Fig. 1e**), whereas the highest-affinity mutant, G5-8, had an affinity of 48 pM (**Fig. 1f**). This affinity represented a 3-million-fold increase relative to the wild-type Vβ8.2:SEB interaction.

To explore whether successive generations of affinity-matured Vβ proteins yielded improvements in neutralizing activity, we performed *in vitro* T-cell assays. In these assays, human class II–positive cells were loaded with ^{51}Cr and incubated with SEB (35 nM), various concentrations of soluble Vβ antagonists and either $Vβ8^+$ cytotoxic T-cell clone 2C (**Fig. 2a**) or polyclonal mouse T cells (**Fig. 2b**). We tested four soluble Vβ proteins: wild-type Vβ8, and representative Vβ proteins from three generations of the affinity maturation process—G2-5, G4-9 and G5-8. As expected, wild-type Vβ8 (micromolar

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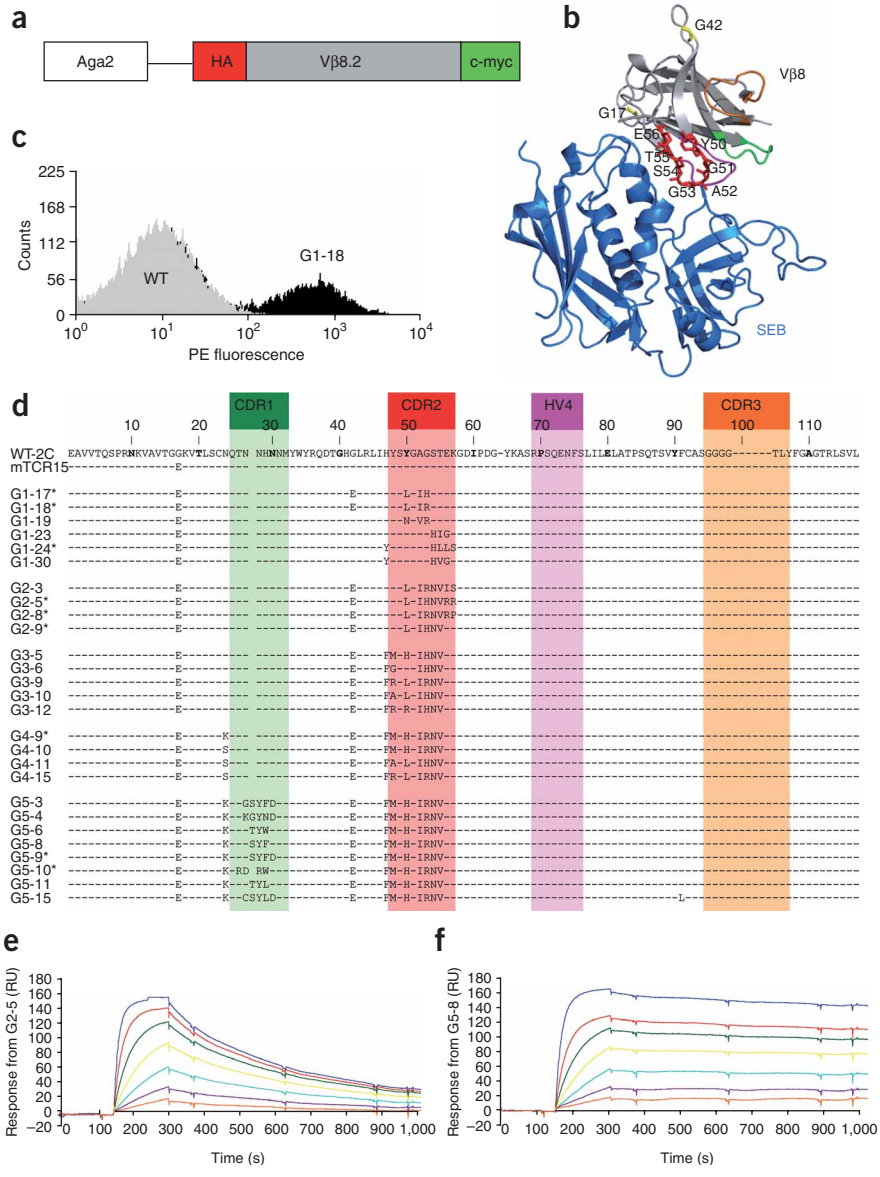

Figure 1 Vβ8 mutants engineered for high-affinity SEB binding. (**a**) Yeast display construct of Vβ8. (**b**) Crystal structure of Vβ8 in complex with SEB. Green, CDR1; red, CDR2; pink, HV4; orange, CDR3. The SEB molecule is shown in blue and Vβ8 residues that contact the SEB molecule are shown in red. Locations of the Vβ-stabilizing residues G17 and G42 are shown in yellow. (**c**) Flow cytometry histogram of the wild-type (WT) Vβ8.2 and the first generation clone G1-18, stained with biotinylated SEB and streptavidin-phycoerythrin (PE). A population of G1-18 did not express the Aga2 fusion and was negative for staining, as has been seen with all yeast-displayed proteins. (**d**) Sequences of Vβ8 mutants from different stages of affinity maturation. G1 through G5 refer to the generation of clones isolated by yeast display. CDR1, CDR2, HV4 and CDR3 regions are highlighted. Clones that were isolated multiple times are indicated with an asterisk. mTCR15 refers to a single-site mutant that had improved display on yeast, compared with the wild-type Vβ8.2 (ref. 8). In the studies described here, we use WT Vβ8.2 to refer to mTCR15, as the latter has only a single amino acid substitution, which does not affect SEB binding but does allow both surface display and expression in *E. coli*. (**e**,**f**) Surface plasmon resonance analysis of affinity-matured Vβ8. Representative SPR sensorgrams of Vβ mutants from generations 2 (G2-5) and 5 (G5-8). Successive dilutions (by a factor of two each time, from 20 nM to 0.3125 nM) of Vβ mutants were analyzed for binding to immobilized SEB (533 response units (RU)). Colors represent different dilutions: blue, 20 nM; red, 10 nM; green, 5 nM; yellow, 2.5 nM; cyan, 1.25 nM; purple, 0.625 nM; orange, 0.3125 nM.

affinity) was ineffective at neutralizing the activity of SEB. In contrast, all three Vβ mutants, which had higher affinity than the wild-type Vβ8, inhibited SEB-mediated activity, with a clear correlation between neutralizing potential and affinity. Previous studies with monoclonal antibodies or soluble receptors for anthrax toxin or botulism toxin have also shown that higher binding affinities are associated with improved inhibitory potential[11–13]. Half-maximal inhibitory concentration (IC_{50}) values were ten times lower for G5-8 ($K_d = 48$ pM) than for G2-5 ($K_d = 650$ pM) (**Supplementary Table 2**). As polyclonal T-cell populations can express different Vβ regions[2], we concluded that the Vβ antagonists were capable of neutralizing SEB-reactive T cells regardless of the expressed Vβ region.

To determine whether Vβ proteins were able to neutralize the activity of SEB *in vivo*, we examined rabbit models of TSS. First we tested the Vβ in an endotoxin-enhancement rabbit model. This model mimics the clinical situation, in which patients with acute-phase TSS have detectable amounts of endotoxin in their sera[14]. Although the role of endotoxin in human TSS is not clear[15], exposure of rabbits to SAgs enhances their susceptibility to endotoxin shock up to 1 million fold[16]. We injected rabbits with 5 μg SEB per kg (body weight), and monitored fever response over 4 h. Rabbits invariably develop fevers within 4 h, and subsequent injection of *Salmonella typhimurium* lipopolysaccharide (LPS) causes death in less than 12 h (ref 16). We injected rabbits intravenously (i.v.) with a combination of 5 μg SEB per kg (body weight) and 500 μg/kg G5-8 Vβ (hereafter referred to as Vβ), or with 5 μg/kg SEB alone (control). We monitored the fever response, and found that rabbits in the control group developed fevers (approximately 2 °C increase), whereas those that received the

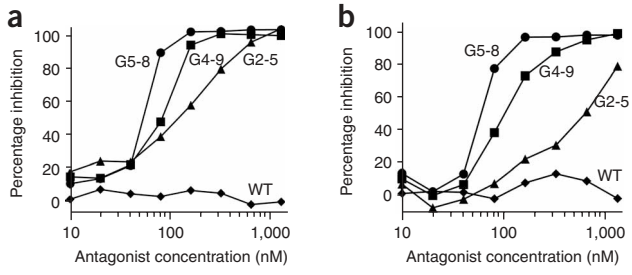

Figure 2 *In vitro* inhibitory activity of soluble, high-affinity Vβ mutants. (**a**,**b**) T-cell inhibitory activity of Vβ mutants in T-cell cytotoxicity assays with clone 2C CTLs (**a**) or polyclonal CTLs (**b**), in the presence of 35 nM SEB and soluble Vβ antagonists.

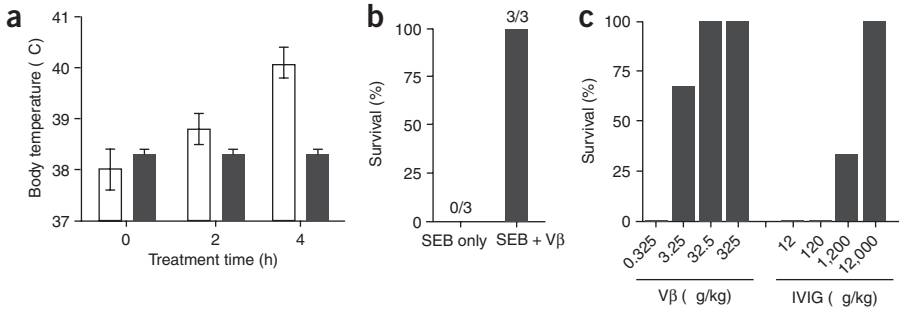

Figure 3 Soluble Vβ blocks the activity and lethality of SEB in rabbits. (**a**) SEB (5 μg/kg) and the fifth generation clone G5-8 (500 μg/kg) were mixed at 24 °C for 1 h. Six New Zealand white rabbits were injected with SEB alone (open bars) or the mixed cocktail (solid bars), and fever response was monitored. (**b**) After 4 h, rabbits were challenged with 100 times the LD_{50} of *S. typhimurium* LPS, and survival was monitored. The number of rabbits that survived treatment is indicated above each bar. (**c**) The experiment described in **a** and **b** was performed with various concentrations of G5-8 Vβ or a high-titer preparation of human IVIG (details in Methods). $n = 3$ rabbits per dose. Percent survival was determined for each group. Error bars represent s.d. (3 rabbits per group).

SEB/Vβ combination had no elevation in temperature (**Fig. 3a**). After 4 h, we injected each rabbit (i.v.) with 0.15 μg/kg LPS, which is 100 times the half-maximal lethal dose (LD_{50}) in rabbits pretreated with 5 μg/kg SEB (the LD_{50} of LPS alone is 500 μg/kg). All rabbits treated with only SEB died, whereas all rabbits treated with SEB/Vβ survived, and with no adverse effects (**Fig. 3b**).

In an independent experiment, we injected three rabbits with one-tenth the amount of neutralizing agent (50 μg/kg Vβ) as that used above, immediately after injection of SEB. As with the higher dose of Vβ, these rabbits exhibited no increase in temperature, and survived. To assess the minimum Vβ dose required for survival, we injected 4 groups of rabbits (3 per group) with SEB (5 μg/kg) and different amounts of Vβ (0.325–325 μg/kg). The Vβ neutralizing agent was partially protective at 3.25 μg/kg (2 of 3 rabbits survived) and completely protective at higher concentrations (6 of 6 survived **Fig. 3c**). We conducted an identical experiment using intravenous immunoglobulin (IVIG, 12–12,000 μg/kg; IVIG is typically administered at 1,000–2,000 μg/kg in the clinic) (**Fig. 3c**). The amounts of Vβ and IVIG required to save half of the rabbits (median effective dose, ED_{50}) were 3 μg/kg and 6,600 μg/kg, respectively. Thus the protective capacity of the Vβ agent was 2,000 times greater than that of IVIG *in vivo*. Additionally, on a molar basis, the Vβ ED_{50} was nearly identical to the SEB concentration used, supporting the notion that the high affinity of this Vβ drives the formation of the inactive Vβ:SEB complexes, even at low Vβ doses.

We next examined whether rabbits with elevated temperatures resulting from SEB exposure could be rescued by treatment with Vβ. We injected rabbits with 5 μg/kg SEB, and 2 h later, after they had developed fevers, with 500 μg/kg Vβ. We found that control rabbits continued to exhibit fevers at 4 h, whereas in Vβ-treated rabbits, temperatures returned to normal levels (**Fig. 4a**). As in the experiment with combined treatment of SEB and Vβ, all rabbits treated with Vβ survived LPS exposure, whereas all control rabbits died (**Fig. 4b**). Surviving rabbits subjected to the same treatment, 1 month after the first, exhibited identical survival results (data not shown), suggesting that induction of antibodies to SEB or Vβ did not influence a secondary exposure to SEB.

The final rabbit model involved a miniosmotic pump system for slow delivery of SEB, mimicking the situation that might be encountered in a staphylococcal infection involving TSS. In this model, pumps containing 200 μg SEB per 200 μl PBS were implanted in rabbits, delivering SEB at a rate of approximately 25 μg/d over 8 d. The experimental group received daily injections of 100 μg Vβ, beginning at the time of implantation. The temperatures of rabbits at time 0 and on day 2 showed that the control rabbits exhibited characteristic fevers whereas the treated rabbits did not (**Fig. 4c**). Control rabbits died of TSS during the 8-d period, whereas treated rabbits survived (**Fig. 4d**).

To gain insight into the *in vivo* action of the Vβ agents, we performed a pharmacokinetic study using radiolabeled Vβ ($[^{125}I]$Vβ) in rabbits. Analysis of the results (**Supplementary Fig. 4** and **Supplementary Table 3** online) showed that the β-phase half-life ($t_{1/2}$) of the Vβ was 325 min, intermediate between that of the V_H or scFv fragments (20–30 min) and that of Fc-bearing antibodies (5 h to several days)[17–21].

It is unclear whether the relatively small size of these Vβ domains (12 kDa) or their serum lifetimes contribute to their *in vivo* effectiveness. The ability of the smaller Vβ proteins to penetrate tissue more effectively than IgG may be useful, especially as the action of SAgs requires cell-to-cell interactions that occur in tissues. The pharmacokinetic studies we performed with the Vβ suggested that its serum lifetime may be adequate to treat individuals with TSS on a daily basis

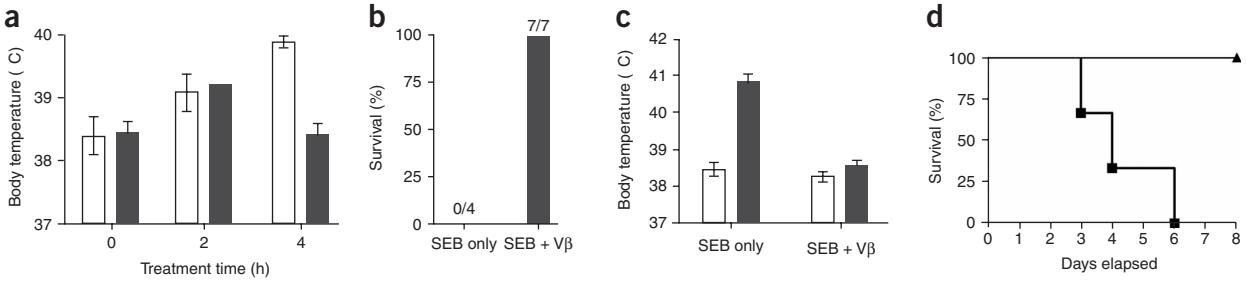

Figure 4 Soluble Vβ rescues rabbits exposed to SEB in the endotoxin enhancement and osmotic pump models. (**a**) SEB (5 μg/kg) and, 2 h later, PBS alone (open bars) or G5-8 (500 μg/kg; solid bars) were administered to rabbits, and fever response was monitored. (**b**) Survival of rabbits challenged with 100 times the LD_{50} of *S. typhimurium* LPS. (**c**) SEB (200 μg) was implanted subcutaneously in two groups of rabbits ($n = 3$ rabbits per group) in Alza miniosmotic pumps. One group of rabbits was given 100 μg G5-8Vβ immediately after implantation, and then daily for 7 d; the other (control) group received PBS. Body temperature was monitored at the time of pump implantation (open bars) and after 2 d of treatment (solid bars). (**d**) Survival of Vβ-treated (triangle) or control (square) rabbits over 8 d.

over a period of a few days. This efficacy of the Vβ, especially in comparison to human IVIG (which may involve additional anti-inflammatory mechanisms[22]), suggests that the high affinity of the Vβ agent may have resulted in an effective treatment even at a dose that was near stochiometric with SEB.

The treatment of SEB-challenged rabbits was successful both when the Vβ was administered after the toxin had already induced elevated temperatures, and when SEB was delivered continuously and daily injections of Vβ were given. This suggests that Vβ agents could be provided on a prophylactic or postinfection basis in a clinical setting.

Staphylococcal and streptococcal infections might involve the presence of multiple toxins requiring neutralization. It is possible that a single Vβ agent could neutralize more than one toxin (for example, the G5-9 Vβ binds SEB and SEC3 with affinities of 93 and 2,500 pM, respectively; data not shown) and that multiple Vβ could be used against different toxins (for example, picomolar-affinity Vβ domains against TSST-1 have been generated[9]). In the future, it should be possible to rapidly detect the presence of specific toxins in a patient, and to tailor a Vβ therapy for neutralizing each of those toxins.

METHODS

Yeast display library construction and screening. The gene encoding mouse Vβ8.2 with the G17E stabilizing mutation was subcloned into yeast display plasmid pCT202 with an N-terminal HA tag and a C-terminal *Myc* tag (**Fig. 1a** and refs. 8,10). Libraries of mutant Vβ were produced by site-directed mutagenesis using overlapping degenerate primers (with NNS codons). Details about library constructions, sizes of libraries and selection parameters are provided in the **Supplementary Note** and **Supplementary Table 1**. Flow cytometry of isolated yeast clones was performed with various concentrations of biotinylated SEB, followed by streptavidin-phycoerythrin (1:500), using a Coulter Epics XL.

Purification and binding analysis of soluble Vβ domains. Soluble Vβ proteins were expressed in BL21(DE3) *E. coli* using the pET28 expression vector (Novagen). Proteins were refolded *in vitro* from inclusion bodies as described previously[8] and purified with Ni agarose resin (Qiagen) or ion exchange chromatography (MonoS column, GE Healthcare); this was followed by gel filtration high-performance liquid chromatography (HPLC) (BioCad Sprint). Proteins were dialyzed against PBS (pH 7.4) before use *in vitro* or in rabbits. We performed surface plasmon resonance (SPR) analysis of Vβ-SEB interactions as described previously[8,9]. We purified Vβ proteins by an additional gel filtration chromatography step, in HBS (pH 7.4), before conducting the binding analysis (Biacore 3000, Biacore). SEB was immobilized by standard amine coupling to a CM5 sensor chip at a density of 500 response units (RU). As a control, we immobilized an equivalent density of TSST-1, which exhibits no detectable binding to Vβ8 or its affinity-matured variants, for all experiments. Proteins were injected for up to 3 min at a flow rate of 25 ml/min and allowed to dissociate for up to 10 min before the binding surfaces were regenerated. Kinetic parameters for association and dissociation were determined using BiaEvaluation 4.1 software (Biacore).

T-cell assays. Daudi, a human lymphoma–expressing class II MHC, was used as the target cell line in cytotoxic T lymphocyte (CTL) assays with SEB. Daudi cells were labeled with ^{51}Cr (MP Biomedicals) and SEB was added to a final concentration of 1 μg/ml (35 nM). Mouse CTL clone 2C was maintained as previously described[23]. Polyclonal CTLs were expanded from Balb/c splenocytes by culturing with 1 μg/ml SEB. Soluble Vβ protein was added, at various concentrations, to effector cells, ^{51}Cr-labeled Daudi cells and SEB (1 μg/ml, 35 nM). After 4 h, we measured the [^{51}]Cr released in supernatants using a gamma counter. We calculated percentage inhibition as [(max c.p.m. – experimental c.p.m.)/(max c.p.m.)] × 100, after subtracting the spontaneous release c.p.m.

Endotoxin enhancement model of toxic shock syndrome. All animal experiments were performed according to protocols approved by the Laboratory Animal Care and Use Committee of the University of Minnesota. We injected rabbits with SEB (i.v., 5 μg/kg) from a 5 μg SEB/ml PBS (0.005 M sodium phosphate, pH 7.2, 0.15 M NaCl) stock, and monitored temperatures hourly for 4 h. At designated time points, we administered intravenous injections of soluble antagonist and 0.15 μg/kg endotoxin (*Salmonella typhimurium*) from a 0.15 μg endotoxin/ml PBS stock. We monitored the rabbits for up to 48 h for signs of TSS and death. Signs of TSS included fever, diarrhea, labored breathing and conjunctival reddening. IVIG preparations (ZLB Bioplasma AG; Immuno AG; and Bayer Healthcare) were used according to the manufacturers' recommendations. For use in rabbits, Vβ and IVIG protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad). We diluted samples in sterile PBS for intravenous injection into marginal ear veins. Doses administered ranged from 0.325 to 325 μg/kg for Vβ and from 12 to 12,000 μg/kg for human IVIG. Rabbits were injected with SEB (5 μg/kg) and then 4 h later with endotoxin (0.15 μg/kg) as above. We recorded deaths over 48 h. To estimate the doses of Vβ and IVIG required for 50% protection, we used the LD_{50} method described in ref. 24.

Miniosmotic pump model of toxic shock syndrome. To assess the ability of Vβ protein to inhibit TSS development during continuous SEB administration, we used a miniosmotic pump model[25]. In this model, rabbits are highly sensitive to the TSS-inducing and lethal effects of SAgs when SEB is continuously released from subcutaneously implanted miniosmotic pumps (Alza)[25]. It has been shown that these pumps release a constant amount of SAg to rabbits over the course of 8 d (ref. 26). In this model, an SEB dose of 200 μg is approximately 4 times the LD_{50}. Young adult rabbits (approximately 2 kg, both sexes) were anesthetized with ketamine and xylazine, and 1-cm incisions were made on the left flanks. A subcutaneous pocket was made in each rabbit, large enough to accommodate the miniosmotic pumps (0.5 cm × 2 cm) loaded with SAgs. The rabbits were sutured, allowed to wake, returned to their cages and monitored for TSS symptoms and death over the course of 8 d. In this model, the highest temperatures generally occur on day 2. Soluble Vβ was administered i.v. in PBS daily.

Coordinates. The PDB code relating to the coordinates of the mouse Vβ8.2 complex with SEB is 1SBB.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

R.A.B. engineered the final generation of Vβ clones, expressed the Vβ proteins in *E. coli*, conducted the *in vitro* neutralization experiments, and helped to draft the manuscript. H.R.O.C. engineered the initial generations of Vβ clones and conducted yeast display experiments. B.M. and E.J.S. conducted the surface plasmon resonance experiments and drafted some sections of the manuscript. M.L.P. conducted the pharmacokinetic studies and evaluated the data. P.M.S. conducted the rabbit experiments, evaluated the data and drafted some sections of the manuscript. D.M.K. helped to design the experiments by R.A.B. and H.R.O.C., evaluated data and drafted the manuscript. All authors provided comments on the final manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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