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## Studies on vascular response to full superantigens and superantigen derived peptides: Possible production of novel superantigen variants with less vasodilation effect for tolerable cancer immunotherapy



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### ABSTRACT

Superantigens (SAGs) are a class of antigens that cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release and causing symptoms similar to sepsis, e.g. hypotension and subsequent hyporeactivity. We investigated the direct effect of SAGs on vascular tone using two recombinant SAGs, SEA and SPEA. The roles of Nitric Oxide (NO) and potentially hyperpolarization, which is dependent on the K<sup>+</sup> channel activation, were also explored. The data show that SEA and SPEA have direct vasodilatory effects that were in part NO-dependent, but completely dependent on activation of K<sup>+</sup> channels. Our work also identified the functional regions of one of the superantigens, SPEA, that are involved in causing the vasodilation and possible hypotension. A series of 20 overlapping peptides, spanning the entire sequence of SPEA, were designed and synthesized. The vascular response of each peptide was measured, and the active peptides were identified. Our results implicate the regions, (61–100), (101–140) and (181–220) which cause the vasodilation and possible hypotension effects of SPEA. The data also shows that the peptide 181–220 exert the highest vasodilation effect. This work therefore, demonstrates the direct effect of SAGs on vascular tone and identify the active region causing this vasodilation. We propose that these three peptides could be effective novel antihypertensive drugs. We also overexpressed, in *E.coli*, four superantigens from codon optimized genes.

### 1. Introduction

Superantigens (SAGs) comprise a large family of disease-associated microbial proteins with potent non-specific T-cell stimulatory activity. SAGs can non-specifically activate up to 20% of resting T-cells, while conventional antigen presentation results in the activation of only 0.001 - 0.0001% of the T-cell population. In this process, SAGs promote various intracellular signal transduction pathways, activating protein kinase C, protein tyrosine kinase (PTK), NF-κB and AP-1 transcription factor signaling, resulting in the release of high levels of specific proinflammatory cytokines by activated T-cells [1]. Subsequently, certain secreted cytokines may attract and activate additional immune cells, including macrophages and polymorphonuclear leukocytes (PMNs) [2]. Activated PMNs may produce excessive amounts of reactive oxygen species (ROS) and release destructive hydrolytic enzymes

that contribute to multi-organ failure and lethal shock.

Patients with infective endocarditis (IE), staphylococcal pneumonia, and some patients after surgery develop sepsis due to infections. It has been shown that SAGs; SEA, TSST-1 and SEC are overexpressed in patients with sepsis compared to patients without sepsis [3–5], raising the possibility that these SAGs contribute to the manifestations of the disease. A recent study confirmed the contribution of SAGs to the development of IE, sepsis and acute kidney injury [6] and *S. aureus* strains that cause sepsis and IE in rabbits, produce TSST-1, SEB, and SEC [7]. The most severe manifestation of sepsis is the septic shock, which is characterized by low systemic vascular resistance and severe hypotension. These abnormal vascular conditions are caused by both excessive vasodilatation and vascular hyporeactivity to circulating catecholamines. The hypotension is often resistant to high doses of catecholamines [8] but can be responsive to vasopressin [9–11] or to inhibition

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of Nitric Oxide (NO) synthase [11]. Excess NO production, through both constitutive and inducible NO synthase isoforms (NOS), low vasopressin secretion and abnormal potassium channel activation [9,12,13], have all been suggested to contribute to the excessive vasodilation associated with this condition. Thus, apart from provoking cytokine release, SAGs appear to stimulate the production of NO, which is a vasodilator produced by the endothelium and in that way induces hypotension and the hyporeactivity characteristic for sepsis. We, therefore, investigated the direct effect of SAGs on vascular tone and the roles of NO and potentially hyperpolarization on the observed effects. This was followed by the determination of the region(s) on the whole superantigen which causes the vasodilatory effect.

Hypertension is a global health problem and is associated with increased mortality of cardiovascular diseases, stroke and diabetes [14]. More than 17.5 million people die every year from cardiovascular disease. Hypertension was projected to affect 29.2% of the global adult population by 2025 [15], and therefore, it has become one of the leading cause of death globally [16].

There are many antihypertensive drugs in the market. In this work; however, we report novel superantigen derived peptides with vasodilatory bioactivity which could be effective antihypertensive drugs.

This work describe the effects of selected SAGs on vascular tone and to shed light on the mechanism of action and to discover the region with the hyporeactivity.

Our study paves the way for in vivo investigation of the novel peptides as antihypertensive drugs and the possible production of superantigen variants with less or no vasodilatory effect for tolerable cancer therapy.

## 2. Material and methods

### 2.1. Bacterial strains, plasmid and chemicals

Prokaryotic IPTG-inducible expression vector, pET28a plasmid and compatible *E. coli* host *Rosetta (DE3)* genetically engineered with T7 polymerase were purchased from Novagen (Madison, WI). DLD-1 was obtained from ATCC collections, DLD-1 (ATCC® CCL221). Ni<sup>2+</sup>-NTA resin was purchased from Sigma-Aldrich (Dorset, England). L. Broth Miller and L. Broth Agar were purchase from Formedia (Devon, England). Kanamycin and chloramphenicol were purchased from Sigma-Aldrich.

### 2.2. Reagents and antibodies

Restriction enzymes and T4 DNA ligase were purchased from Biolabs (London, England) and used as described in the manufacturer's instructions. Standard SEB was purchased from Sigma-Aldrich (labeled as SEBsig throughout the manuscript). Human anti CD3-FITC, anti CD25-PE and Annexin V- FITC were purchased from ImmunoTools (Friesoythe, Germany). All peptides were synthesized and supplied by GenScript.

### 2.3. Codon optimization of SAGs

The genes for SAGs; SEA, SEB, SPEA and TSST-1, were codon optimized for expression in the yeast, *Saccharomyces cerevisiae* (see Results and Discussion sections). Several unique restriction enzyme sites (*Bam*HI, *Nde*I at the 5' end and *Hind*III at the 3' end) were introduced into the gene to facilitate insertion into the cloning vector digested by the same restriction enzymes. The SAG genes, codon optimized for yeast, were synthesized by Geneart GmbH (Regensburg, Germany). The sequences of the codon optimized SAG genes have been deposited in the GenBank database with accession numbers: SEA (KY594411) SEB (KY594412) TSST-1 (KY594413) and SPEA (KY594414).

### 2.4. Sub cloning of SAGs into the expression vector pET28a

The codon optimized SAGs genes were excised by digestion using *Nde*I and *Hind*III and ligated into the identically digested vector pET28a using T4 DNA ligase. Each of the ligation mixtures were transformed into competent *E. coli* and grown on LB media agar plates containing kanamycin (33 µg/ml) at 37 °C. DNA sequence analysis confirmed the correct sequence of the full coding sequence and in-frame fusion to the region of pET28a encoding an N-terminal His-tag. The constructs were then transformed into competent *E. coli Rosetta (DE3)* cells for protein expression studies.

### 2.5. Overexpression of SAGs in *E. coli*

Single colony picks of *Rosetta (DE3)* cells transformed with pETSAGs were grown in LB medium containing the relevant antibiotics (33 µg/ml kanamycin and 34 µg/ml chloramphenicol) at 37 °C until the OD reached 0.45-0.60 at 600 nm. Expression of the SAGs was induced by addition of 1 mM IPTG at 37 °C. After 4 h of induction, the bacterial cells were collected by centrifugation at 4000 rpm for 15 min.

### 2.6. Purification of soluble SAGs using Ni<sup>2+</sup>-NTA chromatography

Recombinant SAGs were isolated from the harvested *E. coli* cells. Cell pellets from 1 l culture were resuspended in 25 ml of 1 mM Tris buffer, pH 7 and sonicated on ice for 5 x 2 min cycles using an MSE Soniprep 150 Plus (1 min sonication pulse followed by 1 min cooling on ice). The soluble protein fraction was separated from the insoluble fraction by centrifugation at 15,000 rpm. The soluble proteins were purified via their N-terminal His-tag by Ni-NTA affinity chromatography. Recombinant SAGs were eluted from the column using 500 mM imidazole containing buffer, pH 7.5. The purified SAGs were dialyzed against 1 mM Tris buffer pH 7, with three exchanges, for 48 h.

### 2.7. Superantigenicity assay

The superantigenicity of purified soluble SAG was determined by treating Peripheral Blood Mononuclear Cells (PBMCs) freshly isolated from human blood using Lymphoprep (Stemcell Technologies, Germany) with the SAGs. In short, PBMCs were seeded (3 × 10<sup>6</sup> cells/well) into 24 well plates and incubated (37 °C, 5% CO<sub>2</sub>) in the presence or absence of the SAG under study (10 µg/ml in 1 mM Tris buffer, pH 7) for 48 h. Culture supernatants were evaluated for cytokines, IL-2, IL-4, IL-5, IL-10 and IFN $\gamma$ , produced by PBMCs in response to SAGs, using a Th1/Th2 Human 5-Plex Panel for Luminex Platform.

### 2.8. MTT assay

To evaluate cell cytotoxicity of superantigens, we prepared PBMCs as above and seeded 5 × 10<sup>5</sup> cells/well in a 96 well plate. Cells were treated with either Media as a control, or with 10<sup>2</sup>ng/ml SAG and 10<sup>4</sup>ng/ml SAG and incubated at 37 °C, 5% CO<sub>2</sub> for 48 h. The cells were then incubated with MTT reagent (5 µg/mL final concentration) at 37 °C for 3 h. 170 µl of DMSO was added into each well to solubilize the formed formazan. The level of formazan production was measured by Infinite M200 PRO Nano Quant Plate Reader at 570 nm.

### 2.9. Assessment of T- Cell activation

To determine T-cell activation, PBMCs, isolated as above, were seeded into a 48 well plate (5 × 10<sup>5</sup> PBMCs/well) and treated with different concentrations (1 ng/ml, 10<sup>2</sup> ng/ml, and 10<sup>4</sup> ng/ml) of SEA, SEB, SPEA, TSST-1 or a commercial preparation of SEB purchased from Sigma-Aldrich (SEBsig). Cells were incubated at 37 °C, 5% CO<sub>2</sub> for 72 h prior to harvesting and staining with anti-CD3 and anti-CD25 antibodies tagged with fluorescent dyes FITC and PE, respectively. T-cell

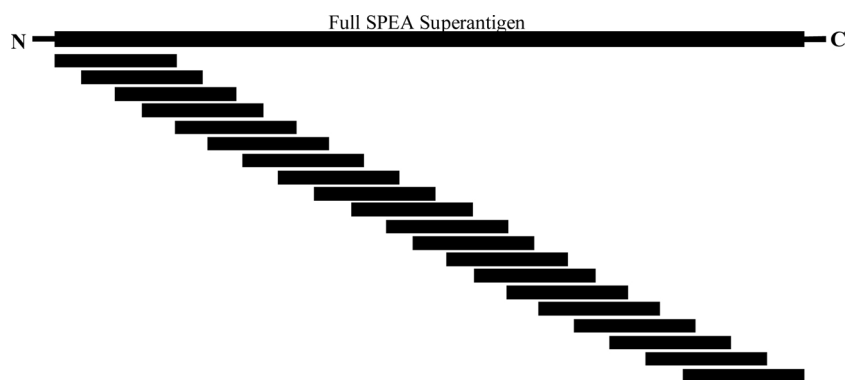


Fig. 1. Design and synthesis of overlapping peptides (SP1 – SP20) based on the SPEA superantigen sequence.

activation analysis was carried out using an Accuri C6 flow cytometer (BD Biosciences) by gating for the lymphocyte subset and measuring the percentage of CD3<sup>+</sup> CD25<sup>+</sup> cells.

### 2.10. Assessment of apoptosis induction

The ability of the SAGs to kill tumor cells was determined using mixed cultures in 48 well plates containing per well  $1.7 \times 10^5$  PBMCs and  $3.5 \times 10^4$  DLD-1 colon tumor cells (obtained from the ATCC: ATCC CCL221). The mixed cultures were incubated at 37 °C in 5% CO<sub>2</sub> for 48 h with different concentrations of SAGs (1 ng/ml, 10<sup>2</sup> ng/ml, and 10<sup>4</sup> ng/ml). After 48 h cells were harvested, stained with Annexin V- FITC and analyzed by flow cytometry.

### 2.11. Synthesis of SPEA based overlapped peptides

We produced peptides covering the complete amino acid sequence of SPEA (Fig. 1 and Table 1). They overlap by thirty amino acids SP1 (1–40), SP2 (11–50), SP3 (21–60), SP4 (31–70), SP5 (41–80), SP6 (51–90), SP7 (61–100), SP8 (71–110), SP9 (81–120), SP10 (91–130), SP11 (101–140), SP12 (111–150), SP13 (121–160), SP14 (131–170), SP15 (141–180), SP16 (151–190), SP17 (161–200), SP18 (171–210), SP19 (181–220), SP20 (191–222).

Table 1

Peptides, overlapping by thirty amino acids and corresponding to the whole amino acid sequence of SPEA, the amino acid in red is the start of the overlapping with the next peptides.

Peptide Name	Sequence	Peptide range
SP1	MQQDPDPSQLHRSSLVKNLQNIYFLYEGDPVTHENVKSVD	1-40
SP2	HRSSLVKNLQNIYFLYEGDPVTHENVKSVDQLLSDHDIYN	11-50
SP3	NIYFLYEGDPVTHENVKSVDQLLSDHDIYNVSGPNYDKLK	21-60
SP4	VTHENVKSVDQLLSDHDIYNVSGPNYDKLKTELKNQEMAT	31-70
SP5	QLLSDHDIYNVSGPNYDKLKTELKNQEMATLFRKDKNVDIY	41-80
SP6	VSGPNYDKLKTELKNQEMATLFRKDKNVDIYVVEYHLCYL	51-90
SP7	TELKNQEMATLFRKDKNVDIYVVEYHLCYLCEAERSACI	61-100
SP8	LFKDKNVDIYVVEYHLCYLCEAERSACIYGGVTNHEGN	71-110
SP9	GVEYHLCYLCEAERSACIYGGVTNHEGNHLEIPKIVV	81-120
SP10	CENAERSACIYGGVTNHEGNHLEIPKIVVKSIDGIQSL	91-130
SP11	YGGVTNHEGNHLEIPKIVVKSIDGIQSLSFDIETNKKM	101-140
SP12	HLEIPKIVVKSIDGIQSLSFDIETNKKMVTAQELDYK	111-150
SP13	KVSIDGIQSLSFDIETNKKMVTAQELDYKVRKYLTDNKQL	121-160
SP14	SFDIETNKKMVTAQELDYKVRKYLTDNKQLYTNGPSKYET	131-170
SP15	VTAQELDYKVRKYLTDNKQLYTNGPSKYETGYIKFIPKKN	141-180
SP16	RKYLTDNKQLYTNGPSKYETGYIKFIPKKNESFWDFPFPE	151-190
SP17	YTNGPSKYETGYIKFIPKKNESFWDFPFPEPEFTQSKYLM	161-200
SP18	GYIKFIPKKNESFWDFPFPEPEFTQSKYLMYKDNELTDS	171-210
SP19	ESFWDFPFPEPEFTQSKYLMYKDNELTDSNTSQIEVYLT	181-220
SP20	PEFTQSKYLMYKDNELTDSNTSQIEVYLT	191-222

### 2.12. Assessment of vascular response

Small arteries were isolated from samples of ovine abdominal muscle obtained from male sheep at the slaughter house in Doha. The arteries were dissected under the LINTRON microscope (BidSpotter, UK) and cut into segments (~2 mm long). Each segment was mounted on 2 wires (40 µm diameter) in an isometric myograph (510 A; JP Trading, Aarhus, Denmark) containing normal physiological salt solution (PSS). The PSS contained 112 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM glucose, bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub> to pH 7.4. The arterial segments were continuously aerated at 37 °C and pre-tensioned to an equivalent of 100 mm Hg (13.3 kPa). The normalized luminal diameter of each segment was obtained as described previously [17]. An equilibration period of at least 1 h was allowed during which tissues were contracted with KCl (90 mM) and noradrenaline (10–100 µM) to optimize tissue response.

Following the equilibration period, the arteries were pre contracted with noradrenaline (10 µM) to achieve a stable tone. After that, SAGs were applied cumulatively, starting from 0.1 µg/ml to a maximum of 10 µg/ml final bath concentrations, to generate dose response curves. Relaxation curves were generated for two of the SAGs (SEA and SPEA). A control curve was also generated for the PBS in which the SAGs were diluted to determine any superantigen unrelated effect. The roles of NO or K ion channels were determined by pretreating the vessels with 100 µM N $\omega$ -Nitro-L-arginine methyl ester (L-NAME, an inhibitor of NO synthase), for at least 30 min or by raising the K ion concentration in the bathing fluid to 35 mM (to block the opening of K ion channels/potential hyperpolarization) prior to applying the superantigens. Data were acquired with a Powerlab acquisition system and LabChart software version 7 (DMT, Denmark). Changes in tone were expressed as the percentage of initial contraction by noradrenaline just before the addition of the lowest concentration of the SAGs. The assessment of the twenty overlapped SPEA based peptides was carried out individually, as mentioned above.

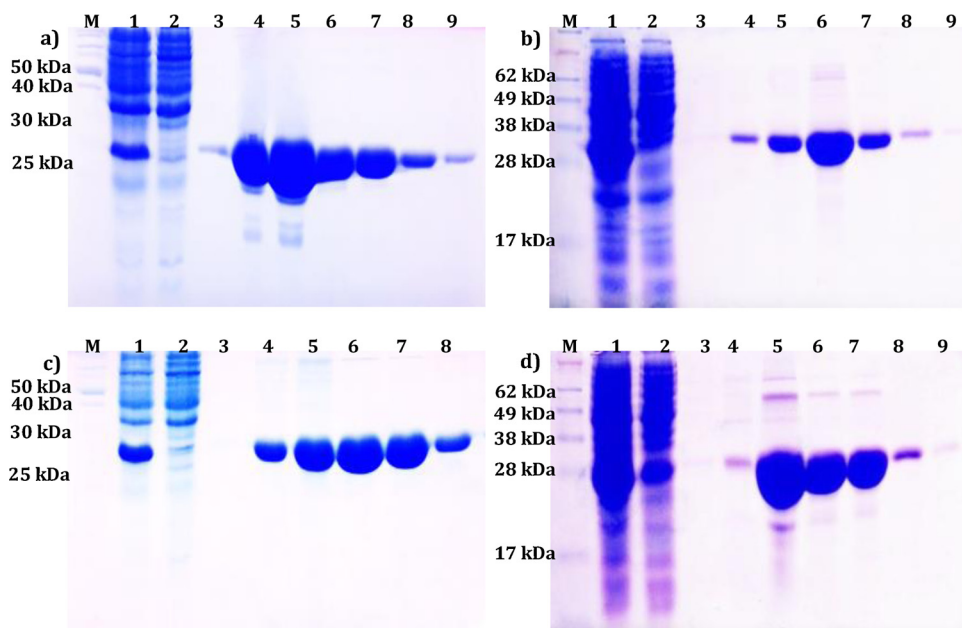
### 2.13. Statistical analyses

Data are presented as mean  $\pm$  standard error of mean (S.E.M) of “n” observations. All graphs were constructed using GraphPad Prism 6 software (San Diego, CA, USA). Statistical analysis was performed using Student’s *t*-test or ANOVA, as appropriate. *P* values < 0.05 were considered statistically significant.

## 3. Results

### 3.1. Overexpression and purification of recombinant SAGs

Codon optimised genes encoding the SAGs SEA, SEB, SPEA and TSST-1 were inserted into expression vector pET28a and transformed



**Fig. 2. Purification of recombinant SAGs expressed from synthetic codon optimized genes.** 10% SDS-PAGE gels of soluble SEA, SEB, SPEA and TSST-1 (Panels a–d, respectively) protein purification. M is Pre-Stained Protein Marker. Lanes 1–3 are total soluble fraction, flow through and wash 5, respectively. Lanes 4–9 are elution fractions.

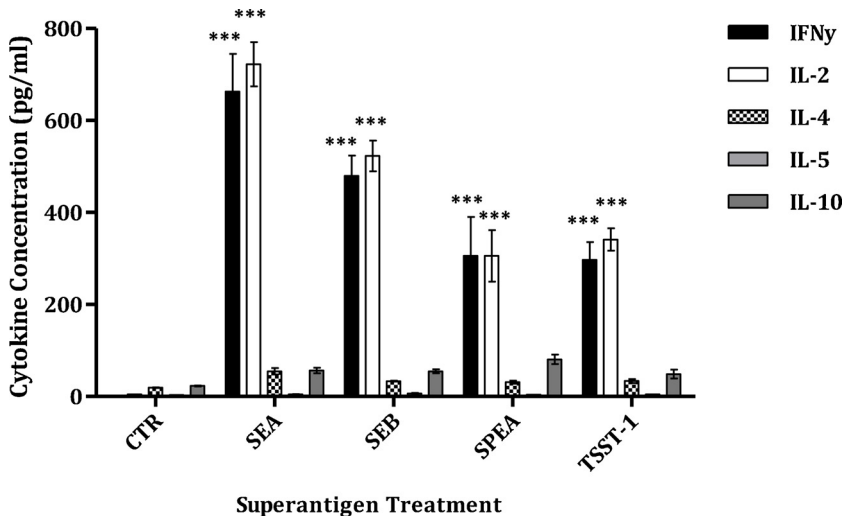
into *E. coli* Rosetta (DE3) cells for overexpression. Following expression, soluble protein fractions were purified by Ni<sup>2+</sup>-NTA affinity chromatography (Fig. 2), which was sufficient to obtain proteins with high purity

### 3.2. Superantigenicity assay

The superantigenicity of purified SAGs was investigated by treating PBMCs with the various SAGs (at 10 µg/ml) or with solvent only prior to measuring the levels of five cytokines, IL-2, IL-4, IL-5, IL-10 and IFN $\gamma$ , excreted into the growth media. As expected while IL-2 and IFN $\gamma$  (hallmarks of superantigenicity) were produced at significantly elevated levels from cells incubated with SAGs (Fig. 3), none of the cytokines was detected in the control. There were no significant levels of IL-5, IL-10 and IL-4 detected. Also, the MTT assay was carried out which shows that SAGs have no cytotoxicity on PBMCs (Fig. 4) and cells proliferation were detected in response to 10<sup>2</sup> ng/ml and 10<sup>4</sup> ng/ml of SAGs.

### 3.3. T-cell activation

The ability of the various recombinant SAGs to activate T-cells was



**Fig. 3. Concentration of IFN $\gamma$ , IL-2, IL-4, IL-5 and IL-10 produced by PBMC cells in response to treatment with 1 mM Tris control, SEA, SEB, SPEA or TSST-1.** Data are presented as mean  $\pm$  standard error of the mean (S.E.M) of 3 independent experiments (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, compared to the buffer control.

investigated. PBMCs were treated with increasing concentrations of SEA, SEB, SPEA, TSST-1 or commercial SEB, prior to staining with fluorescently labeled anti-CD3 and anti-CD25 antibodies and flow cytometric analysis. Fig. 5 shows that both media and buffer controls had no significant effect on T-cells, while up to 20% of T-cells were activated by 1 ng/ml of SAGs SEB, TSST-1 or SEA. All recombinant SAGs dose-dependently increased T-cell activity up to 60%, including SEBsig. There was no significant difference between the standard SEB from sigma (SEBsig) and the recombinant SEB in T-cell activation.

### 3.4. Tumor cell apoptosis

SAGs alone were not efficient in tumor cell killing (Fig. 6), but promoted tumor cell killing by activating T-cells and production of cytokines. Treatment of a mixed culture of PBMCs and DLD-1 tumor cells with different doses of SAG resulted in tumor cell killing up to 80%. Fig. 6 shows a higher percentage of dead tumor cells with higher SAG concentration, (from 1 ng/ml to 10 µg/ml). When a mixed culture of DLD-1 and PBMCs was treated with media or Tris buffer controls, no significant killing was detected. Comparison between SEBsig and recombinant SEB in tumor killing showed no significant difference.

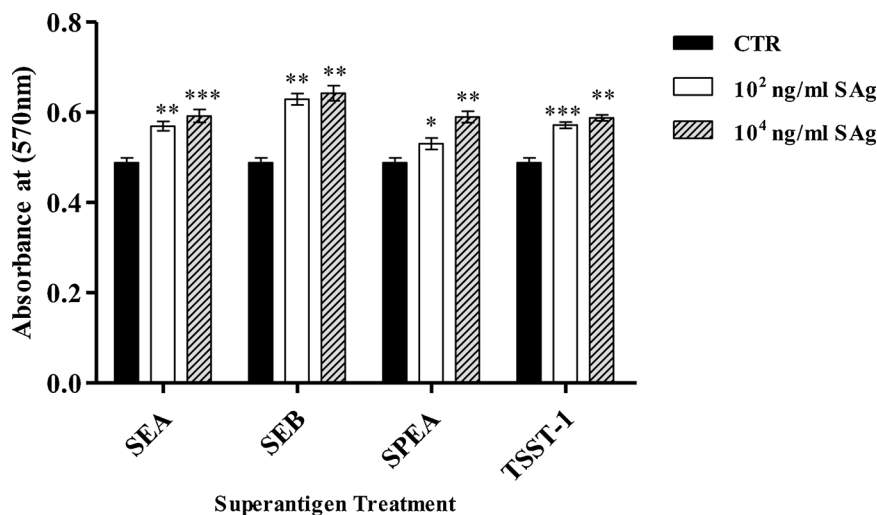


Fig. 4. MTT assay on PBMCs exposed to treatment with different concentrations of SEA, SEB, SPEA or TSST-1 for 48 h. Data are presented as mean  $\pm$  standard error of the mean (S.E.M) of 3 experiments (n = 3). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001, compared to the media control.

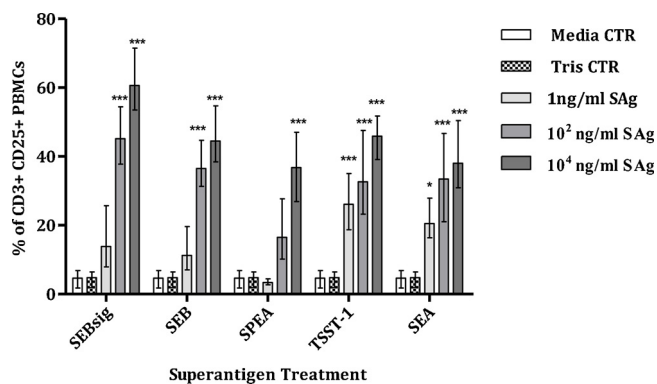


Fig. 5. Percentage of CD3+ CD25+ PBMCs (activated T-cells) in response to treatment with different concentrations of SEBsig, SEB, SPEA, TSST-1, or SEA. Data are presented as mean  $\pm$  standard error of the mean (S.E.M) of 3 independent experiments (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, compared to the buffer control.

3.5. Vascular response to the SAg

A total of 16 ovine skeletal muscle arteries, with the mean normalized internal diameter of  $318 \pm 20 \mu\text{m}$ , were studied for the direct relaxant effects of the SAgS SEA and SPEA. Both SEA and SPEA caused dose-dependent relaxation of these arteries. A typical tracing of the relaxation response to SEA is shown in Fig. 7a and a summary of the responses to both superantigens is shown in Fig. 7b. SEA caused

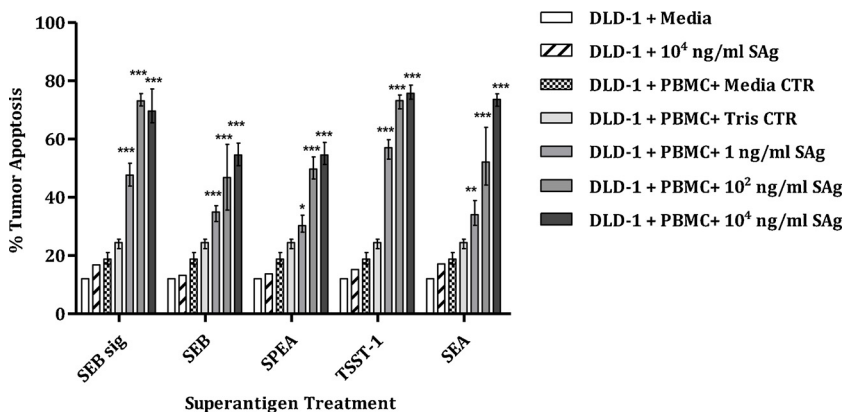


Fig. 6. Percentage of apoptotic tumor cells in response to treatment with different concentrations of SEBsig, SEB, SPEA, TSST-1, or SEA in a mixed culture with PBMCs. Negative controls include: DLD-1 tumor cells with Media, DLD-1 cells with Tris buffer, DLD-1 cells with media, and DLD-1 cells with 10<sup>4</sup>ng/ml of SAg. Data are presented as mean  $\pm$  standard error of the mean (S.E.M) of 3 observations (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, compared to the DLD-1 + PBMC + Tris control sample.

significantly greater relaxation of the arteries compared with SPEA (p < 0.01) in the dose range tested, with  $62 \pm 10\%$  and  $42 \pm 5\%$  relaxation, respectively, following administration of 10  $\mu\text{g/ml}$  of these SAgS.

3.6. Roles of NO and K<sup>+</sup> channel activation in the superantigen-induced vasodilation

Endotoxins can induce the release of NO from the vascular endothelium, which in turn causes relaxation of the underlying vascular smooth muscle. Accordingly, we investigated whether NO also contributes to vasodilation induced by SAgS. In the presence of the NO synthase inhibitor, L-NAME, the relaxation induced by both SEA (Fig. 8a) and SPEA (Fig. 8b) was significantly attenuated (p < 0.01) compared with the respective controls, indicating that NO was involved in their relaxation effects.

To determine whether the relaxation induced by the SAgS also involved the opening of K<sup>+</sup> channels/potential hyperpolarization of the vascular smooth muscle, experiments were carried out in the presence of increased K<sup>+</sup> concentration (35 mM) in the solution bathing the arteries, to prevent opening of these channels. This treatment abolished the relaxation mediated by both SAgS (Fig. 8a and b), suggesting dependence of the SAg-induced relaxation on activation of K<sup>+</sup> channels and potentially hyperpolarization of the vascular smooth muscle.

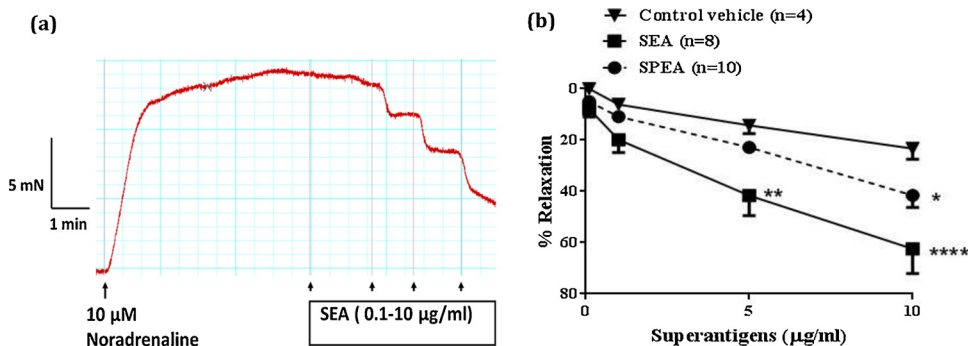


Fig. 7. (a) Typical trace of superantigen-induced relaxation. (b) Vasodilation induced by the two superantigens SEA and SPEA. Both superantigens induced dose-dependent dilation of small skeletal muscle (SKM) arteries. Data are presented as mean ± standard error of the mean (S.E.M). \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001, compared with control vehicle (PBS buffer).

### 3.7. Localization of regions of the SAg SPEA involved in the vascular response

To map region(s) of SPEA involved in the vascular response, we produced a series of peptides spanning the complete amino acid sequence of SPEA. Each peptide overlapped with the next by thirty amino acids (Table 1). The peptides were then tested for their effects on the vascular response as described in the experimental section. SP7 (61–100), SP11 (101–140) and SP19 (181–220) consistently showed marked vasodilator effects above the threshold level, while SP19 showed the highest effect of the three (Fig. 9).

## 4. Discussion

The paper describes the production of recombinant superantigens in *E. coli* from codon optimized genes, and their use to investigate the mechanism of action involved in vascular responses. Although cloning and expression of superantigen genes derived from the chromosomal DNA of the pathogens, has been reported previously, this work used codon optimized synthetic gene versions to produce recombinant superantigens. This approach allowed the large scale production of recombinant superantigens in *E. coli*. Of note, the SAGs genes were codon optimized for expression in yeast but, as we previously serendipitously discovered, this often yields high expression levels of soluble heterologous protein in *E. coli* [18,19]. In the present study, the yeast codon optimized SAGs genes were indeed highly expressed in *E. coli*, with the corresponding proteins accounting for up to 50% of the total protein content after IPTG induction. Purification by Ni<sup>2+</sup> ion column chromatography (Fig. 2) confirmed that each recombinant SAG was equipped with an N-terminal His-tag.

The antitumor effect of the superantigens is believed to be mainly due to their activation of T-lymphocytes, which leads to the production of cytokines and hence to cytotoxicity effects on tumors [20,21]. We

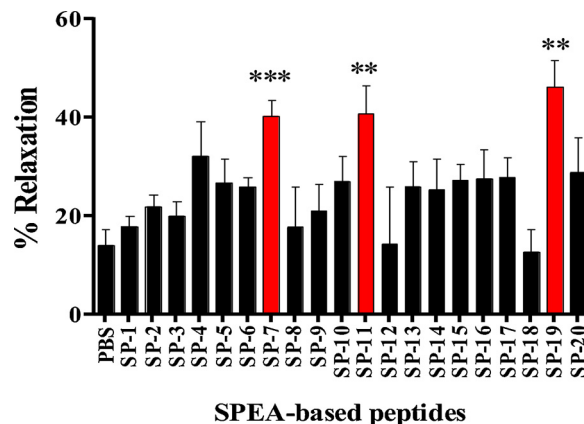


Fig. 9. Identification of peptides derived from superantigen SPEA that induced relaxation. The chart shows the % vasodilation induced by each peptide (n = 5). Three peptides SP7, SP11 and SP19 (red column) induced significant dilation of small skeletal muscle with peptide SP19 showing the greatest effect. Data are presented as mean ± standard error of the mean (S.E.M). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, compared to the buffer control (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

therefore, evaluated the purified recombinant SAGs for their superantigenicity activity towards human PBMCs by measuring proinflammatory cytokines (IL-2, IFN $\gamma$ ). The data shown in Fig. 3 confirmed the induction of IFN $\gamma$  and IL-2. On the other hand Fig. 4 shows that there is no cytotoxicity effect of SAGs on PBMCs. Moreover, the purified recombinant SAGs induced potent T-cell activation (Fig. 5). The T cell activation by the recombinant SAGs was at least 66% of the activation carried out by commercially available SEB.

Subsequently, we evaluated the anticancer activity of the

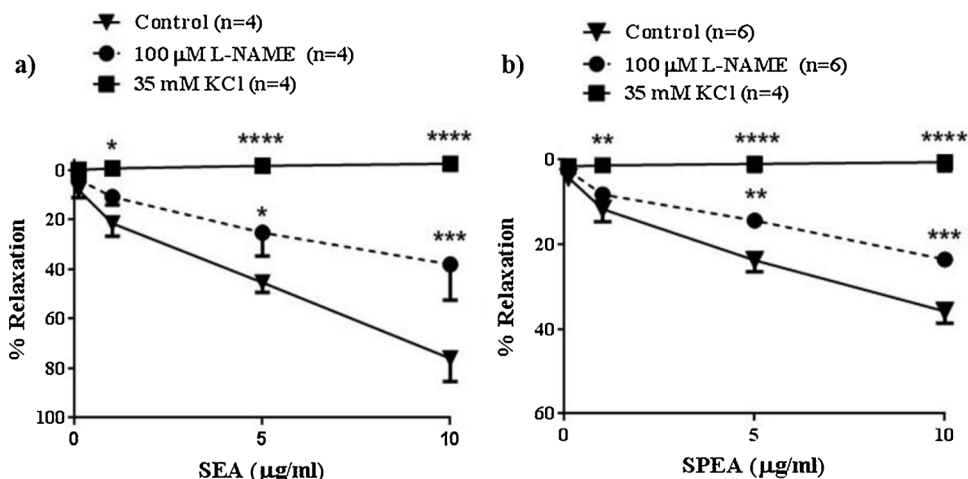


Fig. 8. The effects of 100 μM L-NAME and 35 mM KCl on the relaxation induced by (a) superantigen SEA and (b) superantigen SPEA. Relaxation to these superantigens was partially inhibited by L-NAME and completely abolished by high KCl. Data are presented as mean ± standard error of the mean (S.E.M). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001, compared with control (SAG) responses.

recombinant SAGs. The data in Fig. 6 show the capability of the recombinant superantigens for killing DLD-1 cancer cells through the activation of the T-cell and the production of cytokines. The data in Fig. 6 show that all four recombinant superantigens induced T-cell mediated killing of DLD-1 tumor cells when mixed with PBMCs. In contrast, the recombinant superantigens had no killing effect on tumor cells in the absence of PBMCs (Fig. 6) or killing effect on PBMCs alone (Fig. 4). Collectively, this part of the work demonstrated that recombinant SEA, SEB, SPEA and TSST-1 produced from synthetic codon optimized genes, are biologically active and have potent antitumor capacities.

SAGs have been implicated in some forms of sepsis, which is a major cause of mortality and morbidity. Its most severe manifestation is the septic shock, which is characterized by low systemic vascular resistance and severe hypotension. These abnormal vascular conditions are caused by both excessive vasodilatation and vascular hyporeactivity to circulating catecholamines.

Our data demonstrate that the SAGs, SEA and SPEA have direct vasodilatory effects that are partly NO-dependent, and fully dependent on activation of  $K^+$  channels. Both NO production and potassium channel activation have been implicated in sepsis induced hypotension [22,23].  $K^+$  channels regulate the resting membrane potential of vascular smooth muscle cells and can, therefore, influence vascular tone and blood pressure. The opening of  $K^+$  channels on the arterial smooth muscle membrane allows  $K^+$  to leave the cell and as a result, the cell membrane becomes hyperpolarized. This change in membrane hyperpolarization inhibits the voltage dependent calcium channels, thereby lowering intracellular calcium level and causing arterial dilation [24]. Indeed, abnormal activation of either the ATP-sensitive or the large conductance calcium-activated potassium channels could account for a cardiovascular collapse in shock states [9,25].

The partial role of NO in the SAG-induced relaxation suggests the vascular endothelium also plays a part in these responses. When NO is released from the endothelium, it diffuses into the underlying vascular smooth muscle and activates soluble guanylyl cyclase enzyme to convert guanosine triphosphate to cyclic guanosine monophosphate (cGMP), an important mediator of vasodilation [25].

Due to the above finding, we extended our study in an attempt to identify the region(s) on one of the superantigens, SPEA superantigen that causing vasodilation and therefore possible hypotension. Our results indicate that three peptides, SP7, SP11 and SP19, had a direct vasodilatory effect, with SP19 showing the highest effect (Table 1 and Fig. 9). Previous studies have identifies peptides from milk and other food sources with possible antihypertensive bioactivities [26–31]. Our work presents the isolation of novel superantigen based peptides with vasodilatory effect and possible antihypertensive bioactivity.

The localized dilation of a vessel by peptides as shown in this study does not necessarily lead to the development of systemic changes in blood pressure. Also, previous studies have shown that for peptides, which are taken orally or by intravenous infusion to exert their antihypertensive bioactivity on their targets in vivo they must be absorbed in the intestine and also be resistant to any peptidases degradation [32,33]. Testing in animal models, therefore, is required to establish whether the vasodilation shown by the new peptides could lead to a change in blood pressure and to demonstrate the bioactivity of our new peptides as antihypertensive drugs.

One of the ultimate aims of this part of the study was to produce superantigen variants (with less or no hypotension effect) that have tolerable therapeutic potential, e.g. in cancer immunotherapy. The identification of the regions on superantigen which causes vasodilatory effect could lead to the production of safer superantigen variants to improve the tumor-targeted superantigens (TTSs) technique. TTS is a promising strategy for cancer immunotherapy. Typically, TTSs are constructed by fusing a superantigen to a tumor-specific antibody or ligand that binds to targets that highly or uniquely expressed on cancer cells. TTSs have been applied successfully in several cases [34–40]. This

therapy however, was achieved at the cost of severe side effects, such as high superantigenicity, toxicity and severe hypotension. Our discovery of the region(s) that are implicated in the vasodilatory response, and therefore potential hypotension, may allow the production of novel superantigen variants which lack this region, with less or no vasodilatory effect. These novel superantigen variants could be used for tolerable cancer immunotherapy. Equally, the peptides themselves may be useful starting points for therapies where vasodilation is desirable.

## 5. Conclusion

We show that the SAGs SEA and SPEA induce arteriolar dilatation, which is partly NO-dependent and completely dependent on opening of  $K^+$  channels and potentially hyperpolarization of the vascular smooth muscle, consistent with the potential to cause hypotension. We also identified regions on one of the superantigens, SPEA, which might cause this arteriolar dilatation. Our findings pave the way for the construction and production of superantigen variants with reduced vasodilatory side effects, which could be used for tolerable cancer immunotherapy. Our work may also lead to in vivo investigation of our novel peptides as novel antihypertensive drugs.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Ethics approval and consent to participate

The study was approved by the Anti-Doping Lab-Qatar Institutional Review Board, Ethical approval number: E2017000205. All blood samples used in this study were taken from authors involved in the work.

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