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In Silico Analyses of Staphylococcal Enterotoxin B as a DNA Vaccine for Cancer Therapy

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Abstract Immunotherapy has been suggested as a compelling alternative approach for conventional breast cancer treatment methods. Despite the paramount rolesof T cells in this approach, insufficient numbers of them in the combat against progressive tumor growth still remain to be dealt with. Super antigens are a class of antigens, capable of eliciting T cell proliferation response against desired antigens. Staphylococcal enterotoxin B (SEB) is categorized as a super antigen, its anti-tumor properties has been previously reported. However, to the best our knowledge, SEB has not been ever administered as a DNA construct. In the present study, we exploited bioinformatics tools to assess the immunoreactivity of a SEB-coding DNA construct that serves as a DNA vaccine for breast cancer therapy. Potential B and T (MHC class I and II binders) cell epitopes of the hypothetically expressed protein, along with its sub cellular localization were predicted. Moreover, probable glycosylation and phosphorylation sites within the protein sequence were determined. The gene sequence was optimized according to murine model codon bias and its mRNA stability was analyzed. Employing an integrative in silico approach, we revealed that apparently the construct could be efficiently expressed in mouse model. Moreover, the hypothetically expressed protein could act as an amenable adjuvant in cancer immunotherapy.

Keywords Immunoinformatic · Breast cancer · Adjuvant · Epitope prediction · Immunotherapy · DNA vaccine

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Abbreviations

BCG	*Bacillus* Calmette–Guerin
SAg	Superantigens
SEB	Staphylococcal enterotoxin B
MHC-II	Histocomptability complex class II
TCRs	T cell receptors
PTMs	Post translational modifications

Introduction

Although significant advances have been made in the field of breast cancer treatment, its related mortality and morbidity is still remained high (Siegel et al. 2011). Radiotherapy, surgery and chemotherapy are contemporary approaches for cancer treatment (Berger et al. 2011). Immunotherapy is an alternative modality to struggle against tumor cells (Lanier 2001; Smyth et al. 2001; Sun et al. 2011; Pesonen et al. 2012). Innate and adaptive immunity both are involved in active immunotherapy against cancer. However, executors of adaptive immunity, specially T cells, play more essential roles (Rice et al. 2008; Stevenson et al. 2010).Although tumor-specific T cells have been reported to be elicited in some malignancies, the number of these cells are generally low and insufficient to prevail in combat against a progressive tumor growth (Rosendahl et al. 1996).Hence, promoting these immune responses against tumor cellscould be appreciated. In this regard, bacterial cells and component could be considered as promising agents toarrive at this goal. *Bacillus* Calmette–Guerin (BCG) is the most common agent, used in immunotherapy of bladder cancer(Perabo et al. 2005). However, drastic side effects includingtreatment related deaths have been reported (Lamm et al. 1986). Several researches revealed that bacterial components could be utilized as powerful immunogenic agents with

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direct antitumor activity (Chihara et al. 1969; Pastan et al. 1992; Tokunaga et al. 1999; Melief et al. 2000; Ishii et al. 2003; Borriello et al. 2011; Tsai et al. 2011; Weldon and Pastan 2011; Zhou et al. 2012). It have been demonstrated that toxins are among the bacterial components bearing strong antitumor activities (Patyar et al.2012; Verma et al. 2012). Furthermore, certain bacterial toxins act as super antigens (SAg) (Proft and Fraser 2003).To date, several bacterial Sags were employed by various methods to fight against cancer (Kato et al. 2011; Sun et al. 2011; Xu et al. 2011; Hu et al. 2012). Sags are a class of antigens, possessing the ability to drive powerful non-specific mitogenic activation of T cells (Proft and Fraser 2003; Fraser 2011).

Staphylococcal enterotoxin B (SEB) is a super antigen, subjected to numerous cancer therapy investigations (Fooladi et al. 2009, 2008; Perabo et al. 2005; Fooladi and Nourani 2009; Reis et al. 2011). staphylococcal enterotoxins are a structurally related family of proteins, produced by *Staphylococcus aureus* with similar functional effects (reviewed in (Spaulding et al. 2013)). Their ability to bind both major histocomptability complex class II (MHC II) molecules and T cell receptors (TCRs) in an antigen-processing independent pathway, is the reason behind their similar functional behavior (Proft and Fraser 2003; Ortega et al. 2010). They bind to MHC class II (outside the peptide-binding groove) and variable parts of TCR as an intact protein, and thereby develop a cross-link between these molecules. This triggers signaling cascades which would result in T cell activation and cytokine (particularly TNF-α) secretion. TNF-α is involved in inflammatory response of the body which in normal circumstances (low levels and local release) helps the immune system to deal with pathogens (Serbina et al. 2003). Such responses are suitable in cancer treatment through immunotherapy. However, toxic shock syndrome could be established as a result of sudden and profound T cell stimulation and cytokines production (Proft and Fraser 2003; Fraser 2011). Hence, powerful immune response could act as a double-edged sword. Some disadvantages such as dose-dependence and systemic toxicity of SAgs are the limiting factors for administration of these toxins (Perabo et al. 2005). Streptococcal and staphylococcal enterotoxins are associated with high toxicity, therefore, their administration may result in significant human morbidity and mortality (Perabo 2005). The promising anticancer nature of Sags leads to a growing interest in employment of genetically engineered Sags (Abrahmsén 1995).

To date, several therapeutic SAgs have been engineered to be of reduced toxicity, improved antitumor effects and fused to various proteins (Abrahmsén 1995; Giantonio et al. 1997; Hansson et al. 1997; Alpaugh et al. 1998; LeClaire and Bavari 2001; Erlandsson et al. 2003; Yousefi et al. 2016). DNA vaccination is one of the most attractive and effective immunotherapeutic approaches, employed against cancer (Rice et al. 2008; Stevenson et al. 2010). Introduction of a bacterial toxin as a DNA vaccine could affect structure, function and other properties of the consequently expressed protein (Jahangiri et al. 2011). However, experimental analyses of these effects is expensive, time consuming and in some cases arduous. In silico analyses become a pervasive biological method in prevailing biological studies with noteworthy benefits (Amani et al. 2009; Jahangiri et al. 2012; Khalili et al. 2015).It could be invoked to unveil 3D structure of proteins (Sefid et al. 2013; Jahangiri et al. 2017; Khalili et al. 2017) and vaccine design (Jahangiri et al. 2011, 2017; Khalili et al. 2015; Sefid et al. 2015). Several immunoinformatic tools have been developed to predict B cell and T cell epitopes within the target protein sequences (reviewed in (Khalili et al. 2014)). More precise experimental studies could be conducted based on the results obtained from in silico analyses (Toobak et al. 2013). In the present study, SEB is analyzed as a protein expressed in eukaryotic cells. Hence, the immunological fate of this prokaryotic toxin as a purified protein as well as its toxicity could be differ from one which administrated as a DNA vaccine.In this regard,immunostimulatory effects of SEB-coding DNA construct was thoroughly investigated to be utilized as an adjuvant in cancer immunotherapy studies of murine model. Potential MHC I and II binders of the toxin were predicted. Moreover, linear as well as conformational B cell epitopes were evaluated. Since the construct was hypothetically expressed in eukaryotic cells, potential post translational modifications that could affect immunochemical properties of the recombinant SEB were also reviewed.

Methods

Sequence Retrieval

All analyses were performed on a SEB sequence (Acc. no. YP_185778.1) obtained from "NCBI" at http://www.ncbi.nlm.nih.gov/protein/.

T Cell Epitope Predictions

Several servers were employed to predict potential MHC class I binding peptides within the protein sequence including: "nHLAPred"(Bhasin and Raghava 2007) at http://www.imtech.res.in/raghava/nhlapred/, "ProPred-I" (Singh and Raghava 2003) at http://www.imtech.res.in/raghava/propred1/page2.html, "RANKPEP" (Reche and Reinherz 2007)at http://imed.med.ucm.es/Tools/rankpep and "TmhcPred" at http://www.imtech.res.in/raghava/tmhcpred/ref. Average prediction accuracy of "nHLAPred" is 92.8%(Bhasin and Raghava 2007).This

server invokes a hybrid approach to predict promiscuous MHC class I restricted T cell epitopes(Bhasin and Raghava 2007).The prediction was restricted to murine alleles performed based on combination of artificial neural networks and quantitative matrices. The other options were set as default. "ProPred-I" is a matrix based method which predict MHC binder sequence along with the standard proteasome and immuno proteasome cleavage sites withina given sequence (Singh and Raghava 2003). The murine alleles were selected and the other options were left as default.When threshold is set as 0.5, accuracy of "RANKPEP" predictionsis 60%(Reche and Reinherz 2007).This server predicts MHC class I binders whose C-terminal end is likely to be the result of proteasomal cleavage (Reche and Reinherz 2007). The default setting was not changed. All various lengths of binders restricted to murine alleles were selected for prediction performance.MHC class II binding peptides were predicted by "MHC2Pred" at http://www.imtech.res.in/raghava/mhc2pred/ and "RANKPEP". "MHC2Pred" is an SVM based method with average accuracy of ~80%predicting promiscuous MHC class II binders. All murine alleles available as options of "MHC2Pred" and "RANKPEP" were selected and the other parameters were set as default."CTLpred" (Bhasin and Raghava 2004) at http://www.imtech.res.in/raghava/ctlpred/ and "NetCTL" (Larsen et al. 2007)at http://www.cbs.dtu.dk/services/NetCTL/ were employed for prediction of T cell epitopes. "CTLpred and "NetCTL" integrate prediction of MHC class I binders, proteasomal C terminal cleavage and TAP transport efficiency. The accuracy obtained by "CTLpred"and "NetCTL" predictions are >72% (Bhasin and Raghava 2004; Larsen et al. 2007).Two approaches of "CTLpred" (i.e. Artificial Neural Networks and support vector machine) were harnessed for predicting T-cell epitopes. The remained parameters of settings applied were retained as default. All predicted epitopes and MHC binders were illustrated by DOG 2.0 software(Ren et al. 2009).

Three-dimensional Structure of SEB

Tertiary structure of SEB protein has been experimentally identified (Swaminathan et al. 1992; Papageorgiou; Tranter et al. 1998a, b). Therefore, in order to arrive at the most similar SEB related structures, the SEB sequence served as a query in a "PSI-BLAST"search at http://blast.ncbi.nlm.nih.gov/Blast.cgiagainst protein data bank (PDB). Other parameters of PSI-BLAST were set as default. Tertiary structure of SEB could be employed in conformational B cell epitopes prediction.

B Cell Epitopes Prediction

Prediction of linear B cell epitopes was carried out using "Bepipred" (Larsen et al. 2006) at http://www.cbs.dtu.dk/services/Bepipred/. This server predicts linear B-cell epitopes by a combination of a hidden Markov model and a propensity scale method. Threshold was set as default (0.35). The accuracy of Bepipred predictions is 75% (sensitivity = 0.49 and specificity = 0.75). In order to achieve more reliable linear B cell epitopes, LBtope (Singh et al. 2013) at http://crdd.osdd.net/raghava/lbtope/ was also employed. The accuracy of this method varies from ~54–86% (Singh et al. 2013). This server employs the SVM based models bygenerating dipeptide composition from agiven sequence.Three different models (LBtope_Fixed, LBtope_Variable and LBtop_Confirm models) could be selected.LBtope_Fixed developed on LBtope_Fixed datasetis a 20-mer modelsuitable for predicting fixed length (20 residues) linear B-cell epitopes.The dataset used in this model contain 12,063 unique positive epitopes and 20,589 unique negative ones with 20 residues in length.LBtope_Variable model could predict variable length B-cell epitopes since this model is developed on LBtope_Variable dataset containing 14,876 unique B-cell epitopes and 23,321 unique non B-cellepitopes with variable length.LBtop_Confirm model is developed on those epitopes withvariable length validated at least by two studies (Singh et al. 2013). LBtope_Variable model was used to predict linear B cell epitopes of SEB. Probability of correct epitopes was set as more than 60%. Length of the epitopes was set as 15 residues.

Moreover, several properties including hydrophilicity (Parker et al. 1986), flexibility(Karplus and Schulz 1985), surface accessibility (Emini et al. 1985) and beta-turns (Chou and Fasman 1977) involved in B cell epitope predictions were assessed at http://tools.immuneepitope.org/. Conformational B cell epitopes were predicted by "DiscoTope" (Kringelum, Lundegaard et al. 2012) at http://tools.immuneepitope.org/stools/discotope/discotope.do and "ElliPro" (Ponomarenko. 2008) at http://tools.immuneepitope.org/tools/ElliPro/iedb_input. The average "area under curve" (AUC) performance of "DiscoTope" is >0.727(Kringelum et al. 2012).The "ElliPro"software gives AUC value of 0.732 (Ponomarenko et al. 2008). PDB file obtained from previous step served as an input for both "DiscoTope" and "ElliPro"."Minimum score"for predictingepitopes was set as 0.5 in "ElliPro" prediction procedure. "Maximum distance" for groupingdiscontinuous epitopeswas set as 6 Å.

Codon Optimization

In order to have the highest expression rate of the SEB protein in murine models, "GenScript" service at https://www.genscript.com/sslbin/quote gene synthesiswas invoked for codon optimization of the DNA sequence.

mRNA Stability

Secondary structures and mRNA stability corresponding to the optimized gene were predicted by Mfold at http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form. The folding temperature was at 37 °C. Other parameters were set as default of the server. These properties were compared to that of the mRNA of native gene.

Post Translational Modifications (PTMs)

Possible post translational modifications on the recombinant SEB were predicted by various servers. Servers and their corresponding functions are listed in Table 1.

Prediction of Cell Localization and Signal Peptide

Prediction of the recombinant protein localization within the eukaryotic cells was carried out using servers listed in Table 2. Source of the protein in where the protein localization must be predicted was selected as animal model (for those tools in which the option was available). Presence of any probable signal peptide within the protein sequence was identified by SignalP 4.0 at http://www.cbs.dtu.dk/services/SignalP/.

Table 1 Servers employed for prediction of post translational modifications

Server	Function/prediction	Accuracy (%)	URL	References
NetCGlyc 1.0	Mammalian C-mannosylation sites	93	http://www.cbs.dtu.dk/services/NetCGlyc/	Xu et al. (2011)
NetGlycate 1.0	Predicting glycation of ε amino groups of lysines in mammalian proteins	58	http://www.cbs.dtu.dk/services/NetGlycate/	Yousefi et al. (2016)
NetNGlyc 1.0	*N*-Glycosylation sites in human proteins using artificial neural networks that examine the sequence context of Asn-Xaa-Ser/Thr sequons	76	http://www.cbs.dtu.dk/services/NetNGlyc/	Zhou et al. (2012)
NetOGlyc 3.1	Mucin type GalNAc O-glycosylation sites in mammalian proteins	>75	http://www.cbs.dtu.dk/services/NetOGlyc/	Weldon (2011)
YinOYang 1.2	O-ß-GlcNAc attachment sites in eukaryotic protein sequences	>72	http://www.cbs.dtu.dk/services/YinOYang/	Siegel et al. (2011)
NetPhos 2.0	Serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins	>69	http://www.cbs.dtu.dk/services/NetPhos/	–
NetPhosK 1.0	Kinase specific eukaryotic protein phosphorylation sites	–	http://www.cbs.dtu.dk/services/NetPhosK/	Zhou et al. (2012)
NMT	Myristoylation sites		http://mendel.imp.ac.at/myristate/SUPLpredictor.htm	–

Table 2 Peptide localization predictors used for the protein expression in eukaryotic cells

Server	Accuracy (%)	URL
CELLO (Yu et al. 2006)	87	http://cello.life.nctu.edu.tw/
EpiLoc (Brady and Shatkay 2008)	–	http://epiloc.cs.queensu.ca/
WoLF PSORT (Horton et al. 2007)	>50	http://wolfpsort.org/
PSORT II (Nakai and Horton 1999)	57	http://psort.hgc.jp/form2.html
PROlocalizer (Laurila and Vihinen 2011)	55–97	http://bioinf.uta.fi/PROlocalizer/
SherLoc2 (Briesemeister et al. 2009)	93	http://abi.inf.uni-tuebingen.de/Services/SherLoc2
SLPFA (Tamura and Akutsu 2007)	90.96	http://sunflower.kuicr.kyoto-u.ac.jp/~tamura/slpfa.html
SLP-Local (Matsuda et al. 2005)	>87	http://sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html

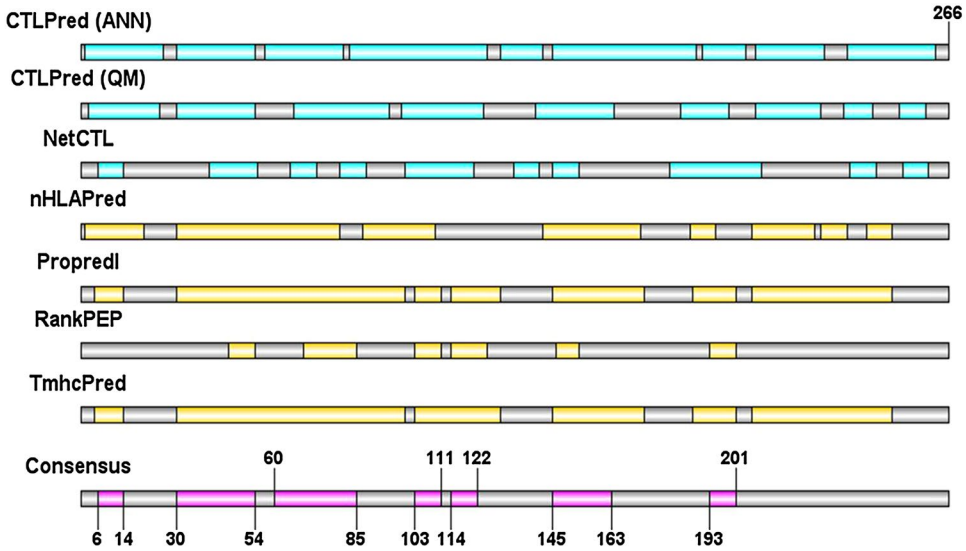

Fig. 1 MHC class I binders-within SEB sequence. The last row shows predicted consensus binders assigned as T cell epitopes. The *gray regions* are unfavorable. The *other colors* show favorable regions. (Color figure online)

Table 3 Amino acid positions of predicted MHC class II binders within SEB sequence

NO.	RANKPEP	MHC2Pred
1	8–19	6–60
2	58–74	66–78
3	76–105	81–94
4	107–125	98–120
5	129–137	126–134
6	148–156	138–197
7	171–179	203–211
8	182–209	223–249
9	228–236	252–267
10	242–256	–
11	258–266	–

Table 4 The first 4 hits with the highest scores of PSI-BLAST searched on the SEB sequence against protein data bank (PDB)

No.	Accession	Total score	Query coverage (%)	*E* value	Identity (%)
1	4RGM_S	233	90	3e−75	99
2	2SEB_D	228	89	2e−73	100
3	1GOZ_A	228	89	3e−73	99
4	3SEB_A	227	89	4e−73	100

Results

T Cell Epitope Predictions

Predicted MHC class I binders acting as T cell epitopes are schematically illustrated in Fig. 1. Peptides were considered as T cell epitopes if"CTLpred" or "NetCTL" and at least three other servers consensually predicted them as epitopes. These epitopes are restricted to murine alleles Table 3 represents predicted MHC II binders restricted to murine model alleles.within SEB sequence.

Three-dimensional Structures

Based on 10 iterations of PSI-BLAST, the highest scored hits bearing most similarity and structural relatedness to the query were obtained. The first four hits with the highest scores of the BLAST are shown in Table 4. The 4th hit with acc. no. 3SEB_A (query coverage: 89%; E value: 4e-73; identity: 100%; total score: 227) was selected as the best sequence with most structural relevance to the query. This hit covered residues 28–265 of the query sequence. PDB file of the sequence was downloaded from PDB data base.

B Cell Epitopes

"Bepipred" and "LBtope" predicted "VLAESQPDPKPDELHK" and "NSHQTDKR" amino acid sequences as linear B cell epitopes with the highest score respectively. Table 5 shows all predicted B cell epitopes by "Bepipred" and "LBtope". Hydrophilic, flexible, surface accessible and beta-turn forming regions of the protein are depicted in Fig. 2. Conformational B cell epitopes predicted by ElliProare listed in Table 6. Figure 3 shows discontinuous B cell epitopes predicted by DiscoTope.

Codon Optimization

GenScriptOptimumGene™ algorithm was employed to optimize various cloning and expression parameters:GC content, codon usage bias, CpG dinucleotides content, mRNA secondary structure, premature PolyA sites, cryptic splicing sites, internal chi sites and ribosomal binding sites, RNA instability motif (ARE), negative

Table 5 Linear B cell epitopes predicted by "Bepipred" and "LBtope" the highest scored epitopes of each server are shown as underlined

Start position	End position	Peptide/bepipred	Start position	End position	Peptide/LBtope
25	40	VLAESQPDPKPDELHK	26	36	LAESQPDPKPD
60	60	V	39	40	HK
83	88	TKLGNY	60	72	VSAINVKSIDQFL
98	106	KDLADKYKD	74	75	FD
126	137	TNDINSHQTDKR	82	82	D
145	154	VTEHNGNQLD	111	115	VFGAN
176	184	QTNKKKVTA	117	138	YYQCYFSKKTN-DINSHQTDKRK
206	211	NSPYET	142	144	YGG
228	237	MPAPGDKFDQ	146	151	TEHNGN
248	252	KMVDS	153	155	LDK
			157	157	R
			168	169	KN
			172	174	SFD
			179	179	K
			181	182	KV
			184	187	AQEL
			227	227	M
			249	253	MVDSK

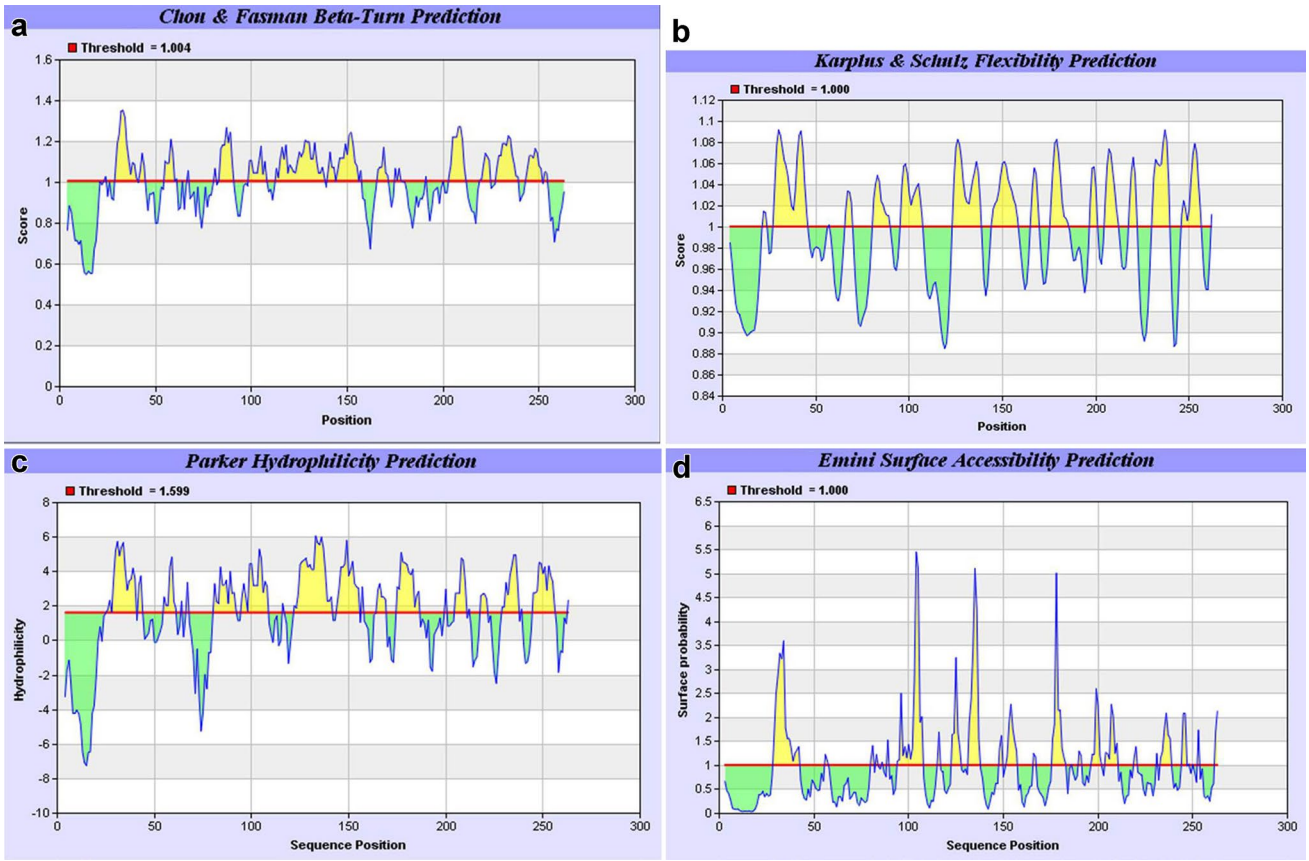

Fig. 2 Plots representing SEB regions with **a** Beta-turn, **b** Flexibility, **c** Hydrophilicity, **d** Surface accessibility properties. These properties are involved in B cell epitope prediction. Horizontal *red line* is the threshold. *Yellow* (above the threshold scores) and *green* (below the threshold scores) areas indicate the favorable and unfavorable regions respectively. (Color figure online)

Table 6 Conformational B cell epitopes predicted by ElliPro

No.	Epitopes predicted by ElliPro	Score
1	A:D42, A:F44, A:L45, A:Y46, A:F47, A:D48, A:I50, A:R65, A:E67, A:Y94, A:F95	0.887
2	A:Q3, A:D5, A:D9, A:L11, A:H12, A:R135, A:F137, A:D139, A:N142, A:T184, A:Y186, A:I190, A:W197, A:D199, A:E231, A:Y233, A:T235, A:T236	0.817
3	A:L20, A:E22, A:N23, A:V26, A:L27, A:E176, A:F177, A:N178, A:N179, A:Q210	0.697
4	A:D29, A:D30, A:V136, A:E138, A:L143, A:L144, A:F146, A:Y167, A:L168, A:V169, A:N171, A:L174, A:Y175, A:Y182, A:V232, A:L234	0.695
5	A:N31, A:V38, A:Q43, A:L49, A:Y51, A:I53, A:D55, A:L58, A:N60, A:Y61, A:V64, A:N88, A:Y90, A:T112	0.692
6	A:F68, A:N70, A:D72, A:L73, A:N220	0.623

The epitopes ranked based on their scores

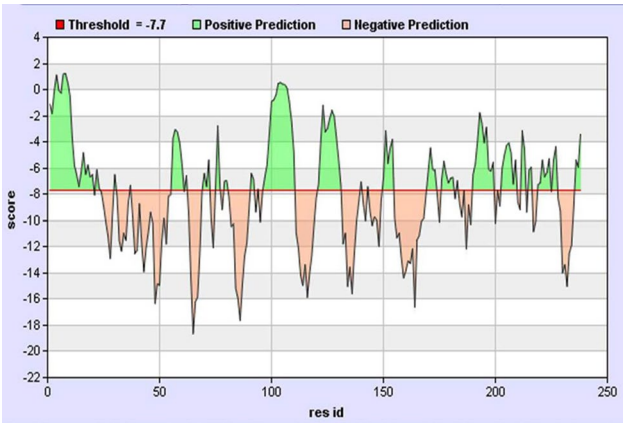

Fig. 3 Prediction of discontinuous B cell epitopes. Residues with a score higher than the threshold (−7.7) which are shown in *green* represent discontinuous B cell epitopes. (Color figure online)

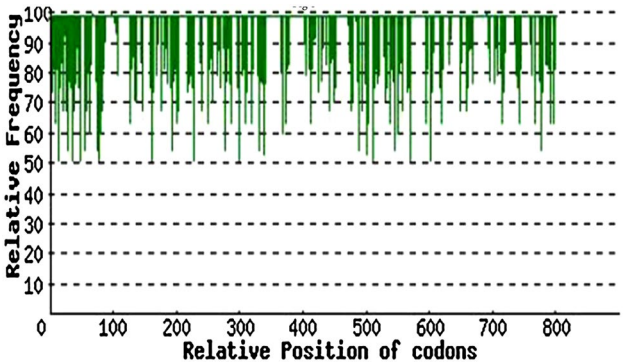

Fig. 4 The distribution of codon usage frequency along the length of the gene sequence. Codon Adaptation Index (CAI) of SEB DNA sequence after optimization to be expressed in mouse is 0.84; CAI of 1.0 is deemed ideal in the desired expression organism. CAI of >0.8 is regarded as good, in terms of high gene expression level

CpG islands, repeat sequences (direct repeat, reverse repeat, and dyad repeat) and restriction sites that may interfere with cloning. These parameters play pivotal roles in gene expression efficiency. Optimized sequence length, including stop codon was 801 bp in length with GC content of 44.17%. The tandem rare codons existed within the native gene sequence could reduce the efficiency of translation or even disengage the translational machinery. Codon Adaptation Index (CAI)was upgraded to 0.84 using mouse codon usage bias (Fig. 4). In addition, to prolong the half-life of the mRNA, unfavorable peaks were optimized. The stem-loop structures, which impact ribosomal binding and stability of mRNA, were broken. Negative *cis*-acting sites were also modified. However, one cis-acting element poly A (AATAAA) at the position of 739 was remained in the optimized gene.Repeated sequences persisting after optimization include: two 9 bp direct repeats with distance of 441 bp, 11 bp inverted repeats at the start positions 552 and 207 with Tm of 36.6, and 10bpdyad repeats at the start positions 625 and 435 (Tm 30.2). Restriction enzymes sites which were filtered within the optimized gene are: *ApaI, BamHI, BglII, EcoRI, HindIII, NcoI, NdeI, NheI, NotI, SacI, SalI, SphI, XbaI* and *XhoI*. There were not any ribosome binding sites as well as polymerase slippage sites after sequence optimization.

mRNA Analyses

Nineteen secondary structures have been predicted for optimized mRNA among which, a model with $\Delta G = -204.70$ kcal/mol possessed the best predicted stability. All predicted structures (except 2 of them) formed U_2-A_{786} and G_3-C_{785} as stack elements. Total ΔG of the two base pairs was −4.30 kcal/mol. Among the 19 models, just two models formed a single stranded structure at the first 16 nucleotides of the 5′ end. The calculated ΔG of these two models was-203.00 and −202.70 kcal/mol. The best structure predicted on native mRNA has Δ Gof −165.10 kcal/mol.

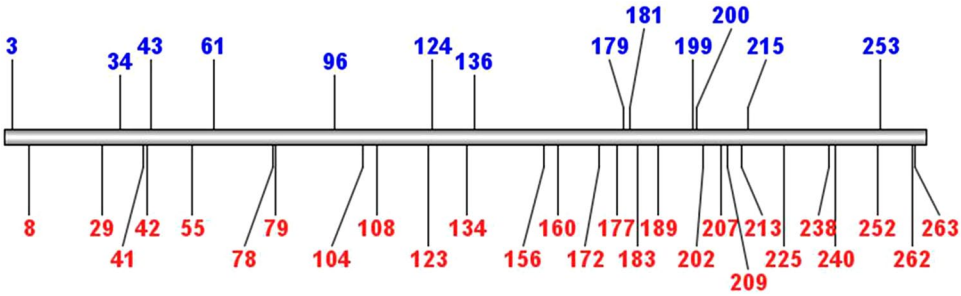

Fig. 5 Residues undergo PTMs (glycosylation and phosphorylation). *Superscript* residues could be glycosylated while the *subscript* ones could be phosphorylated

Table 7 Subcellular localization predictors along with their results on SEB sequence

Server	Predicted localization
CELLO	Nuclear, extracellular, cytoplasmic
EpiLoc	Extracellular
WoLF PSORT	Extracellular
PSORT II	Extracellular
PROlocalizer	ER
SherLoc2	Extracellular
SLPFA	Secretory
SLP-Local	Secretory-pathway

Post Translational Modifications

Thirteen residues within the full-length sequence of the toxin could undergo glycosylation. Amongst, 12 residues were K and one of them was S. No potential myristoylationsite was detected within the protein sequence. Servers employed for determination of phosphorylation sites predicted 27 positions, composed of generic (non kinase-specific) and kinase-specific sites within the sequence. Details on positions of glycosylations and phosphorylations are shown in Fig. 5.

Localization and Signal Peptide

Six out of eight servers predicted secretory pathway or extra cellular localization for the protein sequence expressed in the eukaryotic cells. ER was predicted by one server as subcellular localization of the protein. CELLO server predicted three localizations for the hypothetical protein. Details of predicted localization sites arein Table 7. A signal peptide (1-27aa) with a cleavage site between position 27 and 28 was predicted in both gram-positive bacteria and eukaryotic cells (Fig. 6).

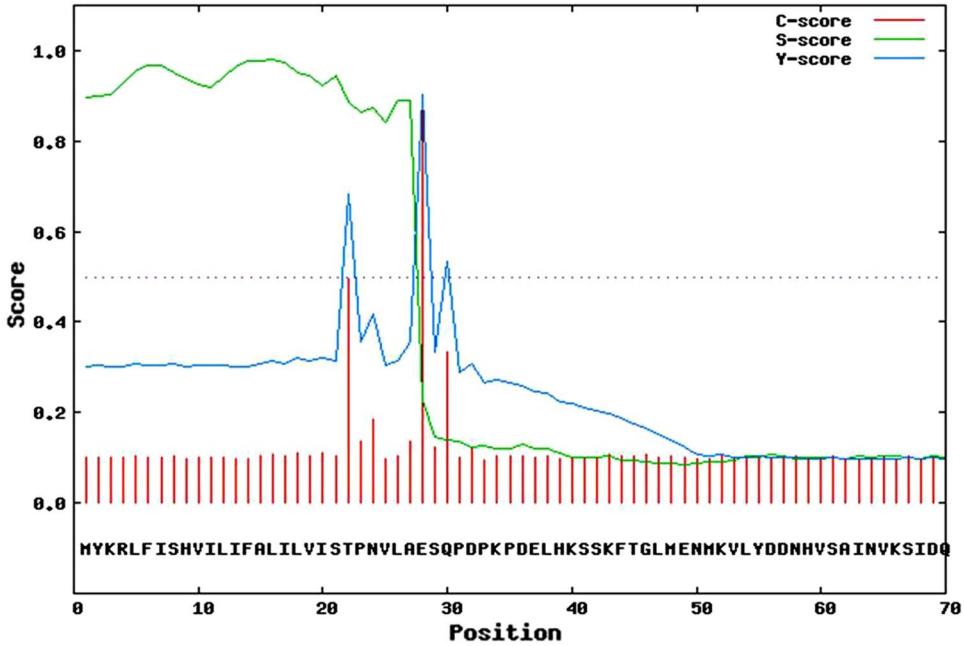

Fig. 6 Signal peptide predicted within SEB sequence. The C-score indicates the first residue in the mature protein. This score is high at the position immediately after the cleavage site. S-score distinguishes between positions within signal peptide from those of the mature part of the proteins. Y-score is a geometric average of the C-score and the slope of the S-score. (Color figure online)

Discussion

Super antigens are a class of toxins employed in immunotherapy against cancer. Anti-cancer activity of SEB, a member of group I SAgs, has also been fairly demonstrated(Perabo et al. 2005; Fooladi et al. 2008; Fooladi and Nourani 2009; Reis et al. 2011). This toxin could elicit responses and proliferations of both $CD4^+$ and $CD8^+$ T cells (Fleischer and Schrezenmeier 1988).However, some disadvantages and side effects are coupled withthe administration of protein toxins. Prevalence of anti-staphylococcal enterotoxin antibodies in human serum may potentially inhibit T-cell activation (LeClaire and Bavari 2001). Moreover, prolonged exposure to the toxin would elicit IL-10 and consequently deletion or anergy of the activated T cells. Production of IL-2, MHC class II and co-stimulatory molecules on the surface of APCs may be reduced by IL-10. These events lead immune system toward production of memory cells, unresponsive to antigen stimulation (Lussow and MacDonald 1994; Miller et al. 1999). $CD8^+$ T cells are relatively resistant to the anergy, while this can mainly affect $CD4^+$ T cells (Perabo et al. 2005). In the present study we attempt to evaluate effects of a SEB-derived DNA construct as an adjuvant for cancer immunotherapy via an in silico approach. The protein translated from a DNA construct could have different fatesin eukaryotic cells. Intact protein could be bound to MHC class II and TCR in an antigen-presentation independent pathway (reviewed in (Spaulding et al. 2013)). Administration of a DNA construct could expose T cell epitopes of SEB to host immune system. These T cell epitopes include MHC class II and I binding peptides which are normally inaccessible to immune system. Secretory pathway (extracellular)was predicted as probable subcellular localization of the hypothetically expressed protein in eukaryotic cells. It could be due to existence of the signal peptide with bacterial origin within the full-length protein. Based on in silico analysis, this sequence could be also identified as a signal peptide in a eukaryotic cell. This peptide would most likely be removed during protein folding and maturation process. Preexisting anti-SEB antibodies can interfere with its function and therapeutic effects (Erlandsson et al. 2003). It is unclear whether antibodies raised against wild type SEB could successfully recognize SEB, expressed by eukaryotic cells. However, antibody responses may be efficiently induced by full-length protein and secreted in to immune system. Recently, four monoclonal antibodies (mAbs), recognizing conformational B cell epitopes of native SEB, have been described (Varshney, Wang et al. 2011). Residues 135-R, 137-F, 186-Y, 188-K, 231-E, 233-Y and235, 236-Tin mature SEB are essentially important for interactions of SEB with previously mentioned mAbs (Varshney et al. 2011). Interestingly, these epitopes are actively involved in the formation of the second conformational epitope, predicted by ElliPro. It implies that predicted epitope could be recognized by four previously introduced mAbs. Since these antibodies recognize conformational epitopes, structural alteration could lead tofailure in antibody/antigen interactions. Bacterial antigens, targeted to secretory pathway by DNA vaccination could be affected by eukaryotic glycosylation (Jahangiri et al. 2011). Therefore, the impact of probable glycosylations on conformation, function and immunochemical properties of the expressed protein must be taken in to account. Glycosylation is a common post translational modification, common lyoccurs in eukaryotic cells. It has been demonstrated that protein glycosylation is also occurs in bacteria. However, exerted pattern of the modifications is different from eukaryotic cells (reviewed in (Nothaft and Szymanski 2010)). The first residue, predicted to be glycosylated in the eukaryotic cells, is located in position 3 within the leader peptide sequence. Since the signal peptide would be removed, its modification would not have any major impact on the conformation of the mature secreted protein. Positions 34, 136, 179 and 181are located within regions predicted as linear B cell epitopes, while residues located in positions 43, 124, 199, 200 and 253 are those involved in conformational B cell epitopes. Linear and conformational epitopes have the 136 and 179 residues in common. Since prokaryotes and eukaryotes have different patterns of post translational modifications, pre-existed antibodies against native toxin could not recognize those epitopes in the recombinant protein. So, interference with its adjuvant and therapeutic properties could be alleviated. Hence, the probable post translational modifications could be considered an advantage from an immunochemical point of view. Other researchers reduced affinity of antibodies against SEA and SEE using genetic removal of antibody binding sites around MHC class II binding sites of these super antigens (Erlandsson et al. 2003). Secreted protein could be presented in two main ways: (1) MHC class II presentation pathway (activation of $CD4^+$ T cells pathway) and (2) cross-presentation by the activation of$CD8^+$ T cells (reviewed in (Rice et al. 2008)).SEB can directly bind and trigger a signal transduction pathway through TCR, and then stimulate T-cell proliferation without any requirement to a specific presenting molecule(Perabo et al. 2005). The affinity of SAg for TCR determines its biological ability for antibody response stimulation. The higher the affinity is, the stronger the responses would be elicited (Arcus et al. 2000). Amino acids responsible for TCR activation within the full-length sequence of protein, are L47, E49, N50, V53, L54, D56, D57, N58, V60, D82, L85, N87, Y88, A114, N115, Y116, Y117, Y118, Q119, T139, F204, Q237 and L241(Papageorgiou et al. 1998a, b). None of these amino acids would be glycosylated in a eukaryotic cell. Thus, glycosylation could not directly interfere with

SEB–TCR interaction, albeit, the structure and conformation of the protein could be affected. However, amino acid K96 involved in MHC class II interaction would be glycosylated. In the case of SEA, decreased affinity of the toxin to MHC class II (with mutation or fusion to an antibody) would result in less toxicity (Forsberg et al. 2001; Erlandsson et al. 2003). Therefore, glycosylation of the residue number 69 would decrease toxicity of SEB, attenuating SEB–MHC class II interactions. Phosphorylation of proteins could result in increased efficiency of their degradation (Dougan et al. 2012), promoting MHC class I-binders presentation. Moreover, this modification could have influences on SEB structure and its interaction with MHC class II as well as TCR. As a hypothesis, these posttranslational modifications could weaken SEB–TCR and SEB–MHC class II interactions. Thus, the toxicity and T cell activation properties of the recombinant SEB would be reduced. It would be expected that administration of this DNA vaccine could lead to tumor size reduction. However, the hypothesis must be validated by precise practical evaluations. Expression level of the recombinant protein could be regulated by gene optimization as well as vector engineering. Gene and mRNA analyses revealed that the optimized SEB gene could be efficiently expressed in eukaryotic cells.

In conclusion, performed analyses indicate that administration of SEB as DNA vaccine could trigger moderate T cell expansion without significant toxicity. Thus, it could serve as a useful adjuvant in immunotherapy of cancers.

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Compliance with Ethical Standards

Conflict of interest No conflict of interest declared.

References

Abrahmsén L (1995) Superantigen engineering. Curr Opin Struct Biol 5(4):464–470

Alpaugh RK, Schultz J et al (1998) Superantigen-targeted therapy: phase I escalating repeat dose trial of the fusion protein PNU-214565 in patients with advanced gastrointestinal malignancies. Clin Cancer Res 4(8):1903–1914

Amani J, Mousavi SL et al (2009) In silico analysis of chimeric espA, eae and tir fragments of *Escherichia coli* O_{157}:H_7 for oral immunogenic applications. Theor Biol Med Model 6:28

Arcus VL, Proft T et al (2000) Conservation and variation in superantigen structure and activity highlighted by the three-dimensional structures of two new superantigens from *Streptococcus* pyogenes1. J Mol Biol 299(1):157–168

Berger B, Stahlberg K et al (2011) Impact of radiotherapy, chemotherapy and surgery in multimodal treatment of locally advanced esophageal cancer. Int Soc Cell 81(5–6):387–394

Bhasin M, Raghava G (2004) Prediction of CTL epitopes using QM, SVM and ANN techniques. Vaccine 22(23):3195–3204

Bhasin M, Raghava G (2007) A hybrid approach for predicting promiscuous MHC class I restricted T cell epitopes. J Biosciences 32(1):31–42

Borriello M, Laccetti P et al (2011) A novel fully human antitumour immunoRNase targeting ErbB2-positive tumours. Br J Cancer 104(11):1716–1723

Brady S, Shatkay H (2008) EpiLoc: a (working) text-based system for predicting protein subcellular location. In: Pacific Symposium on Biocomputing. World Scientific, pp. 604–615

Briesemeister S, Blum T et al (2009) SherLoc2: a high-accuracy hybrid method for predicting subcellular localization of proteins. J Proteome Res 8(11):5363–5366

Chihara G, Maeda Y et al (1969) Inhibition of mouse sarcoma 180 by polysaccharides from Lentinus edodes (Berk.) sing. Nature 222:687–688

Chou PY, Fasman GD (1977) Secondary structural prediction of proteins from their amino acid sequence. Trends Biochem Sci 2(6):128–131

Dougan DA, Micevski D et al (2012) The N-end rule pathway: from recognition by N-recognins, to destruction by AAA+proteases. Biochim et Biophysica Acta (BBA)—Mole Cell Res 1823(1):83–91

Emini EA, Hughes JV et al (1985) Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J Virol 55(3):836–839

Erlandsson E, Andersson K et al (2003) dentification of the antigenic epitopes in staphylococcal enterotoxins A and E and design of a superantigen for human cancer therapy. J Mol Biol 333(5):893–905 I.

Fleischer B, Schrezenmeier H (1988) T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells. J Exp Med 167(5):1697

Fooladi AAI, Nourani M (2009) Histological analysis antimetastatic effect of intera-venus injection of staphylococcal enterotoxin b and monophosphoryl lipid a against fibrosarchoma in lung tissue. J Iran Anat Sci 7:121–131

Fooladi AAI, Sattari M et al (2008) In vivo induction of necrosis in mice fibrosarcoma via intravenous injection of type B staphylococcal enterotoxin. Biotechnol Lett 30(12):2053–2059

Fooladi, I. A. A., Sattari M et al Synergistic effects between staphylococcal enterotoxin type B and monophosphoryl lipid A against mouse fibrosarcoma. J BUON 15(2): 340

Forsberg G, Ohlsson L et al (2001) Therapy of human non-small-cell lung carcinoma using antibody targeting of a modified superantigen. Br J Cancer 85(1):129

Fraser JD (2011) Clarifying the mechanism of superantigen toxicity. PLoS Biol 9(9):e1001145

Giantonio BJ, Alpaugh RK et al (1997) Superantigen-based immunotherapy: a phase I trial of PNU-214565, a monoclonal antibody-staphylococcal enterotoxin A recombinant fusion protein, in advanced pancreatic and colorectal cancer. J Clinical Oncol 15(5):1994–2007

Hansson J, Ohlsson L et al (1997) Genetically engineered superantigens as tolerable antitumor agents. Proc Nat Acad Sci USA 94(6):2489

Horton P, Park K-J et al (2007) WoLF PSORT: protein localization predictor. Nucleic Acids Res 35(suppl 2): W585–W587

Hu J, Xuan X et al (2012) Anti-tumor function of double-promoter regulated adenovirus carrying SEA gene, in the treatment of bladder cancer. Cell Biochem Biophys: 1–7

Ishii KJ, Kawakami K et al (2003) Antitumor therapy with bacterial DNA and toxin. Clin Cancer Res 9(17):6516–6522
Jahangiri A, Rasooli I et al (2011). An in silico DNA vaccine against listeria monocytogenes. Vaccine 29:6948-6958
Jahangiri A, Rasooli I et al (2012). Precise detection of L. monocytogenes hitting its highly conserved region possessing several specific antibody binding sites. J Theor Biol
Jahangiri A, Rasooli I et al (2017) In silico design of an immunogen against *Acinetobacter baumannii* based on a novel model for native structure of Outer membrane protein A. Microb Pathog 105:201–210
Karplus P, Schulz G (1985) Prediction of chain flexibility in proteins. Naturwissenschaften 72(4):212–213
Kato M, Nakamura Y et al. (2011) Enhanced anti-tumor immunity by superantigen-pulsed dendritic cells. Cancer Immunol Immunother 60:1–10
Khalili S, Jahangiri A et al (2014) Computational vaccinology and epitope vaccine design by immunoinformatics. Acta Microbiol Immunol Hung 61(3):285–307
Khalili S, Rahbar MR et al (2015) In silico analyses of Wilms tumor protein to designing a novel multi-epitope DNA vaccine against cancer. J Theor Biol 379:66–78
Khalili S, Jahangiri A et al (2017) Structural pierce into molecular mechanism underlying *Clostridium perfringens* epsilon toxin function. Toxicon. doi:10.1016/j.toxicon.2017
Khalili S, Rasaee MJ et al (2017) 3D structure of DKK1 indicates its involvement in both canonical and non-canonical Wnt pathways. Mol Biol 51(1):155–166
Kringelum JV, Lundegaard C et al (2012) Reliable B cell epitope predictions: impacts of method development and improved benchmarking. PLoS Comput Biol 8(12):e1002829
Lamm D, Stogdill V et al (1986) Complications of *Bacillus* Calmette–Guerin immunotherapy in 1278 patients with bladder cancer. J Urol 135(2):272
Lanier LL (2001) A renaissance for the tumor immunosurveillance hypothesis. Nature Med 7(11):1178–1180
Larsen J, Lund O et al (2006) Improved method for predicting linear B-cell epitopes. Immunome Res 2(2):1–7
Larsen MV, Lundegaard C et al (2007) Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. BMC Bioinformatics 8(1):424
Laurila K, Vihinen M (2011) PROlocalizer: integrated web service for protein subcellular localization prediction. Amino Acids 40(3):975–980
LeClaire RD, Bavari S (2001) Human antibodies to bacterial superantigens and their ability to inhibit T-cell activation and lethality. Antimicrob Agents Chemother 45(2):460–463
Lussow AR, MacDonald HR (1994) Differential effects of superantigen-induced "anergy" on priming and effector stages of a T cell-dependent antibody response. Eur J Immunol 24(2):445–449
Matsuda S, Vert J-P et al (2005) A novel representation of protein sequences for prediction of subcellular location using support vector machines. Protein Sci 14(11):2804–2813
Melief, C. J. M., R. E. M. Toes et al (2000) Strategies for immunotherapy of cancer. Adv Immunol 75:235–282
Miller C, Ragheb JA et al (1999) Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms in vivo. J Exp Med 190(1):53
Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends Biochem Sci 24(1):34–35
Nothaft H, Szymanski CM (2010) Protein glycosylation in bacteria: sweeter than ever. Nat Rev Microbiol 8(11):765–778
Ortega E, Abriouel H et al (2010) Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. Toxins 2(8):2117–2131
Papageorgiou AC, Tranter HS et al (1998a) Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. J Mol Biol 277(1):61
Papageorgiou AC, Tranter HS et al (1998b) Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 Å resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors. J Mol Biol 277(1):61–79
Parker J, Guo D et al (1986) New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. BioChemistry 25(19):5425–5432
Pastan I, Chaudhary V et al (1992) Recombinant toxins as novel therapeutic agents. Annu Rev Biochem. doi:10.1146/annurev.bi.61.070192.001555
Patyar S., A. Prakash et al. (2012) Bacteria as a therapeutic approach in cancer therapy. in: Bacteria and cancer. Springer, Dordrecht, pp. 185–208
Perabo, F. G. E., Willert PL et al (2005) Preclinical evaluation of superantigen (staphylococcal enterotoxin B) in the intravesical immunotherapy of superficial bladder cancer. Int J Cancer 115(4):591–598
Pesonen S, Diaconu I et al (2012) Oncolytic immunotherapy of advanced solid tumors with a CD40L expressing replicating adenovirus: assessment of safety and immunological responses in patients. Cancer Res 72:1621–1623
Ponomarenko J, Bui H-H et al (2008) ElliPro: a new structure-based tool for the prediction of antibody epitopes. BMC Bioinform 9(1):514
Proft T, Fraser JD (2003) Bacterial superantigens. Clin Exp Immunol 133(3):299–306
Reche PA, Reinherz EL (2007) Prediction of peptide-MHC binding using profiles. Methods Mol Biol 409:185–200
Reis LO, Ferreira U et al (2011) Anti-angiogenic effects of the superantigen staphylococcal enterotoxin B and *Bacillus* Calmette–Guérin immunotherapy for nonmuscle invasive bladder cancer. J Urol 187:438-445
Ren J, Wen L et al (2009) DOG 1.0: illustrator of protein domain structures. Cell Res 19(2):271–273
Rice J, Ottensmeier CH et al (2008) DNA vaccines: precision tools for activating effective immunity against cancer. Nat Rev Cancer 8(2):108–120
Rosendahl A, Hansson J et al (1996) Immune response during tumor therapy with antibody-superantigen fusion proteins. Int J Cancer 68(1):109–113
Sefid F, Rasooli I et al (2013) In silico determination and validation of baumannii acinetobactin utilization a structure and ligand binding site. BioMed Res Int. doi:10.1155/2013/172784
Sefid F, Rasooli I et al (2015) Functional exposed amino acids of BauA as potential immunogen against *Acinetobacter baumannii*. Acta Biotheor 63(2):129–149
Serbina NV, Salazar-Mather TP et al (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity 19(1):59–70
Siegel R, Ward E et al (2011) Cancer statistics, 2011.CA 6:212–236
Singh H, Raghava G (2003) ProPred1: prediction of promiscuous MHC Class-I binding sites. Bioinformatics 19(8):1009–1014
Singh H, Ansari HR et al (2013) Improved method for linear B-cell epitope prediction using antigen's primary sequence. PLoS ONE 8(5):e62216
Smyth MJ, Godfrey DI et al (2001) A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol 2(4):293–299
Spaulding AR, Salgado-Pabón W et al (2013) Staphylococcal and streptococcal superantigen exotoxins. Clin Microbiol Rev 26(3):422–447

Stevenson FK, Ottensmeier CH et al (2010) DNA vaccines against cancer come of age. Curr Opin Immunol 22(2):264–270

Sun Z, Shi L et al (2011) Immune modulation and safety profile of adoptive immunotherapy using expanded autologous activated lymphocytes against advanced cancer. Clin Immunol 138(1):23–32

Swaminathan S, Furey W et al (1992) Crystal structure of staphylococcal enterotoxin B, a superantigen. Nature 359(6398):801–806

Tamura T, Akutsu T (2007) Subcellular location prediction of proteins using support vector machines with alignment of block sequences utilizing amino acid composition. BMC Bioinformatics 8(1):466

Tokunaga T, Yamamoto T et al (1999) How BCG led to the discovery of immunostimulatory DNA. Jpn J Infect Dis 52(1):1

Toobak H, Rasooli I et al (2013) Immune response variations to *Salmonella enterica* serovar Typhi recombinant porin proteins in mice. Biologicals 41(4):224–230

Tsai AK, Oh S et al (2011) A novel bispecific ligand-directed toxin designed to simultaneously target EGFR on human glioblastoma cells and uPAR on tumor neovasculature. J Neuro-Oncol 103(2):255–266

Varshney AK, Wang X et al (2011) Generation, characterization, and epitope mapping of neutralizing and protective monoclonal antibodies against staphylococcal enterotoxin B-induced lethal shock. J Biol Chem 286(11):9737–9747

Verma RS, Potala S et al (2012) Application of microbial toxins for cancer therapy. Microorganisms in sustainable agriculture and biotechnology. Springer, Dordrecht, pp. 647–662

Weldon JE, Pastan I (2011) A guide to taming a toxin–recombinant immunotoxins constructed from Pseudomonas exotoxin A for the treatment of cancer. FEBS J 278(23):4683–4700

Xu M, Wang X et al (2011) An engineered superantigen SEC2 exhibits promising antitumor activity and low toxicity. Cancer Immunol Immunother 60(5):705–713

Yousefi F, Siadat SD et al (2016) Tagging staphylococcal enterotoxin B (SEB) with TGFaL3 for breast cancer therapy. Tumor Biol 37(4):5305–5316

Yu CS, Chen YC et al (2006) Prediction of protein subcellular localization. Proteins 64(3):643–651

Zhou X, Qiu J et al. (2012). "In vitro and in vivo anti-tumor activities of anti-EGFR single-chain variable fragment fused with recombinant gelonin toxin. J Cancer Res Clin Oncol 138:1–10