Preparation and In Vitro Evaluation of Antitumor Activity of TGFαL3-SEB as a Ligand-Targeted Superantigen

Forough Yousefi, MSc¹, Seyed Fazlollah Mousavi, PhD¹, Seyed Davar Siadat, PhD¹, Mohammad Mehdi Aslani, PhD¹, Jafar Amani, PhD², Hamid Sedighian Rad, MSc², and Abbas Ali Imani Fooladi, PhD²

Abstract
Tumor-targeted superantigens (TTSs) have been used to treat a variety of tumors in preclinical studies. The TTS utilizes the powerful T-cell activation strategy by means of staphylococcal enterotoxins (SEs) as superantigens (Sags) to target tumor cells. Monoclonal antibodies and tumor-related ligands have been used as targeting molecules of Sag. In this study, we assessed the antitumor potency of tumor-targeted superantigen (TTS) strategy to design and produce fusion protein as a new antitumor candidate. The third loop (L3) of transforming growth factor (TGF) was genetically conjugated to staphylococcal enterotoxin type B (TGFαL3-SEB), and its in vitro antitumor activity against murine breast cancer cells (A431 cell line) was evaluated. We designed and prepared TGFαL3-SEB chimeric protein and evaluated superantigenic activity, binding property to cancer cells, over-expression of epidermal growth factor receptor (EGFR), and in vitro antitumor activities. Cloning of tgfαl3-seb was confirmed by colony-polymerase chain reaction, enzymatic digestion, and sequencing. The recombinant TGFαL3-SEB fusion protein with molecular weight of 31 kDa was expressed and confirmed by anti-His Western-blot analysis. The TGFαL3-SEB fusion protein attached to A431 cell line with proper affinity and induced dose-dependent cytotoxicity against EGFR-expressing cancer cells in vitro. The TGFαL3-SEB chimeric protein exhibited potent in vitro antitumor activity. Our findings indicated that TGFαL3-SEB may be a promising anticancer candidate in cancer immunotherapy, and further studies are required to explore its potential in vivo therapeutic applications.

Keywords
breast cancer, cloning and expression, immunotherapy, staphylococcal enterotoxin type B (SEB), transforming growth factor (TGF-α)

Abbreviations
ATP, adenosine triphosphate; EGFR, epidermal growth factor receptor; HRP, horseradish peroxidase; hTGF-α, human transforming growth factor α; IPTG, isopropyl-β-D-thiogalactoside; L3, third loop; LTTs, ligand-targeted therapeutics; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; Ni-NTA, nickel-nitrilotriacetic acid; OD, optical density; PCR, polymerase chain reaction; PHA, phytohemagglutinin; RPMI, Roswell Park Memorial Institute medium; Sags, superantigens; SEs, staphylococcal enterotoxins; SEB, staphylococcal enterotoxin type B; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEA, staphylococcal enterotoxin A; TCGI%, percentage of tumor cell growth inhibition; TcR, T-cell receptor; TGF-α, transforming growth factor α; TGFαL3-SEB, third loop of transforming growth factor α genetically conjugated to staphylococcal enterotoxin type B; TTS, tumor-targeted superantigens

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¹ Bacteriology Department, Pasteur Institute of Iran, Tehran, Iran
² Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

Corresponding Authors:
Abbas Ali Imani Fooladi, PhD, Applied Microbiology Research Centers, Baqiyatallah University of Medical Sciences, Tehran, Iran. Email: imanifouladi.a@gmail.com; imanifouladi.a@bmsu.ac.ir
Seyed Fazlollah Mousavi, PhD, Bacteriology Department, Pasteur Institute of Iran, Tehran, Iran. Email: sadatff@yahoo.com; mousavi@pasteur.ac.ir
Introduction

Breast cancer is the most common cancer in women\(^1\)-\(^4\) and has been increasing in men.\(^5\) Although advances in screening, surgery, adjuvant radiation, and systemic therapies are in practice, there is no effective cure for patients with advanced stages of the disease.\(^6\) Therefore, the development of novel treatments that can increase the survival of the patients with breast cancer is welcomed.

Activation of the patients’ immune system is one of the several promising therapeutic methods for controlling progression of cancer because tumor cells often avoid presenting their antigens to T cells. One of the major goals of tumor immunotherapy is generating tumor-specific T cells that finally contribute to the eradication of tumors.\(^7\) Advances have been made to understand the role of the host immune responses along with alternative treatments in intervening tumor progression.\(^8\)

Superantigens (SAgs) are bacterial and viral proteins that share the ability to activate a large number of T lymphocytes. Bacterial SAgs bind to the class II major histocompatibility complex (MHC) antigens expressed on professional antigen-presenting cells outside the peptide-binding groove and consequently bind to the T-cell receptor (TcR) via the variable region of the TcR \(\beta\)-chain.\(^9\)-\(^11\) Superantigens are efficient inducers of inflammatory cytokine production and cell-mediated cytotoxicity.\(^12\)-\(^15\)

Currently purified bacterial products are gaining relevance as new classes of bioactive products to treat and prevent cancer growth and metastasis.\(^16\) Staphylococcal enterotoxins (SEs) are powerful SAgs that activate all T cells expressing a defined set of V\(\beta\)-TCR, irrespective of their actual antigen specificity.\(^7\) Enterotoxins are produced by Staphylococcus aureus and draw considerable attention as ideal agent for cancer therapy.\(^17\)

Tumor-targeted superantigens (TTS) represent a novel concept for cancer immunotherapy which aim to activate and provoke T lymphocytes to attack tumor cells. These objectives can be achieved by means of fusing bacterial Sags to Fab fragments of tumor-reactive monoclonal antibodies (mAbs) or ligands that bind to receptors that are either uniquely expressed or overexpressed on the target cells relative to normal tissues. This allows a specific delivery system of antitumor agent to the cancer cells. Ligand-targeted therapeutics (LTTs) have advantages to mAbs. Tumor-related ligand is less antigenic than mAbs, plus nonantibody ligands are often readily available, inexpensive to manufacture, and easy to handle\(^18\) and facilitate drug penetration into solid tumors.\(^19\)

In this study, we chose the staphylococcal enterotoxin B (SEB) as bacterial Sags that is a potent inducer of cytotoxic T-cell activity and cytokine production in vivo.\(^7\)

Variation in the regulation and expression of growth factors and/or their receptors has been correlated with the development and prognosis of malignancies.\(^20\) Generally, the targeted antigen or receptor should have a high density on the surface of the target cells,\(^18\) so we chose the epidermal growth factor receptor (EGFR) as a suitable target for the design of LTTs in breast cancer immunotherapy.

The EGFR is a commonly expressed 170-kDa transmembrane glycoprotein that is a member of the HER tyrosine kinase growth factor receptor family and is involved in signaling pathways affecting cellular growth, proliferation, and differentiation.\(^20\) The EGFR has been implicated in the pathogenesis of multiple human tumors. Overexpression of the receptor has also been noted frequently in breast, bladder, and ovarian tumors as well as in various squamous carcinomas.\(^21\) The EGFR is generally reported as an adverse prognostic marker.\(^22\)-\(^24\) Moreover, the degree of EGFR overexpression has been correlated with advanced tumor stage and resistance to standard therapies. High levels of receptor expression have been found in 30% to 40% of carcinomas.\(^21\) The EGFR is encoded by the proto-oncogene, \(c\)-erb-B.\(^25\)-\(^27\) Activation of this proto-oncogene results in overexpression of EGFR in many human tumors.

The EGFR is composed of an extracellular ligand-binding region that is the ligand-binding site for both the epidermal growth factor (EGF) and transforming growth factor \(\alpha\) (TGF-\(\alpha\)),\(^20\) a lipophilic transmembrane anchor sequence, and an intracellular domain characterized by an adenosine triphosphate (ATP)-dependent tyrosine kinase.\(^25\)-\(^28\),\(^29\) Human transforming growth factor \(\alpha\) (hTGF-\(\alpha\)) is a native ligand, co-overexpressed with its receptor EGFR in many human tumors.\(^30\) When ligand binds, receptor dimerization occurs and the intracellular tyrosine kinase domain triggers subsequent signaling pathways involved in cell growth regulations and survival. The hTGF-\(\alpha\) consists of 3 loops, the third of which (TGF\(\alpha\)L3) retains the binding ability to EGFR.\(^20\) Ligand–receptor internalization occurs upon binding of the intact ligand to its receptor; however, the Sag functions extracellularly and its function is independent of its cellular uptake. In this study, TGF-\(\alpha\) was selected as a ligand to block induced ligand–receptor internalization, and SEB Sag was fused to the third loop of TGF-\(\alpha\) (TGF\(\alpha\)L3). This will not only retain the binding ability to EGFR but also prevent the ligand/receptor internalization. Moreover, TGF\(\alpha\)L3 is presumably less antigenic compared to mAbs; thereby, it has a longer half-life in vivo. These interesting properties of TGF\(\alpha\)L3 make it an attractive targeting molecule for the Sags toward tumors.\(^30\)

Here, we designed and constructed TGF\(\alpha\)L3-SEB chimeric protein, and its binding property to EGFR-expressing cancer cells was determined in vitro. Also, its antitumor activity on 4T1 murine breast cancer cell line was evaluated. The 4T1 cells grow as adherent epithelial cells in vitro and are used as a model of murine estrogen-nonresponsive mammary carcinoma cells. When injected into Balb/c mice, 4T1 cells multiplied rapidly resulting in highly metastatic tumors and served as an animal model for human stage IV breast cancer.\(^31\),\(^32\) Interestingly, we found that the TGF\(\alpha\)L3-SEB fusion protein could bind to EGFR-expressing tumor cells with proper affinity and exhibited an apparent inhibitory effect on the growth of 4T1 tumor cells in vitro.
Table 1. Characteristics of Published Primers, Nucleotide Sequences, and PCR Programs.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5'-3'</th>
<th>PCR Programa</th>
<th>Amplicon Size, bp</th>
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<tr>
<td>nuc detection</td>
<td>F: ATGGCATTACGTAATGTTTCG</td>
<td>1</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>R: TTTAGGATCTGGTTTCAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seb detection</td>
<td>F: TCCGATCAAATTGACAAC</td>
<td>2</td>
<td>495</td>
</tr>
<tr>
<td></td>
<td>R: GCAGGTACTCTGATAATGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seb cloning</td>
<td>F: AGCAGAATTCATGTGAGTCAACAG</td>
<td>3</td>
<td>720</td>
</tr>
<tr>
<td></td>
<td>R: TAACGAAGCTTTCACTTTCGGGTCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: F, forward; PCR, polymerase chain reaction; R, reverse.

1. 30 times (94°C, 1 minute; 55°C, 1 minute; 72°C, 1 minute); 2. 30 times (94°C, 1 minute; 64°C, 45 seconds; 72°C, 1 minute); and 3, 35 times (94°C, 1 minute; 63°C, 1 minute; 72°C, 1 minute).

Material and Method

Material and Reagent

Restriction endonucleases and silica-based DNA gel extraction kits were obtained from Fermentas Thermo Fisher Scientific, Inc (Waltham, Massachusetts). Nickel-nitrilotriacetic acid (Ni-NTA) column, polymerase chain reaction (PCR) purification kit, and DNA extraction kit were purchased from Qiagen GmbH (Germany), Metabion (Germany), and Bioneer (Korea), respectively. Deoxyadenosine triphosphate was obtained from Fermentas. The 10× thermostable buffer, pfu DNA polymerase, and MgSO₄ were purchased from Promega (Germany).

Nitrocellulose membrane and diaminobenzidine were purchased from Schleicher (USA) and Sigma (Germany), respectively.

Oligonucleotides used as primers for PCRs and tgfz-linker sequence were synthesized by Bioneer and ShineGene Molecular Biotech, Inc (China), respectively.

Bacterial Strains and Cell Culture

Staphylococcus aureus was isolated from clinical samples. Escherichia coli strain TOP10 was used for plasmid propagation and cloning and E coli strain BL21 (DE3) as a host for the production of fusion proteins. pET-28a+ was used as expression vector, supplied by Novagen Inc (Madison, Wisconsin).

A431 human epidermal carcinoma cell line (NCBI C204) and 4T1 mouse breast cancer cell line (NCBI C604) were purchased from Pasteur Institute of Iran and were maintained in Dulbecco Modified Eagle medium (Gibco; Life Technology, Maryland) and Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco; Life Technology), respectively. The media were supplemented with 10% fetal bovine serum (Gibco, Life Technology), 100 units/mL penicillin, and 100 mg/mL streptomycin.

Mouse spleen cells, freshly separated from healthy BALB/c mice, were grown in RPMI cell culture medium.

Optimization of the PCR for Detection of seb-Positive S aureus Strain

In this study, 200 clinical specimens were screened for detecting seb-positive S aureus isolates. All biochemically tested S aureus were confirmed by PCR using specific primers for S aureus thermonuclease (nuc gene), then PCR was performed to screen for seb-positive strains. Staphylococcus aureus isolates were grown in 10 mL brain heart infusion broth at 37°C overnight under aerobic conditions. Bacterial cells were collected and DNA was extracted with DNA extraction kit based on kit manual. The amplification reaction was performed in a final volume of 25 μL, containing 1 μL genomic DNA, 0.5 μL Taq polymerase (5 unit/μL), 1 μL each of the primers (10 pmol/μL), 2 μL each of 2.5 mmol/L deoxy ATP, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxyguanosine triphosphate, 2.5 μL of 10× PCR buffer (50 mmol/L KCl), 10 mmol/L Tris-HCl (pH 8.3 at 25°C), and 1.5 μL MgCl₂ (50 mmol/L). Primer sequences and PCR programs used for detection of nuc and seb are shown in Table 1. The amplified products were analyzed by electrophoresis with a 1% agarose gel followed by ethidium bromide staining and ultraviolet transilluminator visualization. The PCR products were verified by sequencing.

Design and Construction of Chimeric Gene

To design TGFzL3-SEB chimeric construct, we chose 17 amino acids from the N-terminus of third loop of transforming growth factor α (TGFzL3). This sequence was reported to be involved in binding to the receptor, EGFR. The 239 amino acids of the full length of mature SEB (accession: KC428707.1) were selected and fused to the C-terminus of the chimeric fusion protein. The 2 fragments were bridged by a linker consisting of 8 amino acids (GGSGSGGGG). The amino acid composition of TGFzL3 and SEB sequences was computed using bioinformatics databases and tools (Figure 1).

Cloning of seb and tgfzL3-seb Genes

The 720-bp DNA sequence encoding the mature SEB from seb-positive S aureus was amplified by PCR using specific primers that included 5’ EcoRI and 3’ HindII restriction enzyme sites, respectively, to facilitate cloning (Table 1). For PCR amplification, the reaction mixture (30 μL) contained 1 μL of each of the primers (10 pmol/μL), 1 μL of deoxynucleotide triphosphate mixture (2.5 mmol/L each), 3.0 μL of 10× PCR buffer,
isolated recombinant constructs SEB and TGFα.

Individual clones were grown at 37°C using the program shown in Table 1. The amplified were subjected to thermal cycling (Eppendorf Mastercycler preparation was added to each 0.2-mL reaction tube. The tubes (5 units/centrifugation, and the resulting pellets were then frozen at 

Expression Optimization and Purification of TGFαL3-SEB Fusion Protein and SEB

Expression host E. coli TOP10 competent cells and cultured onto Luria–Bertani (LB) medium, containing 100 mg/mL ampicillin at 37°C. The PCR-selected clones were confirmed by restriction digestion and DNA sequencing.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

The recombinant proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The SDS-PAGE was carried out using 12.5% gels. Recombinant proteins were solubilized in reducing sample buffer. Polyacrylamide gels were stained by Coomassie blue G-250 to detect the protein. Gels were blotted onto a 0.45-μm nitrocellulose membrane, and then nonspecific binding sites were blocked using 5% bovine serum albumin (BSA) dissolved in 0.1 mol/L PBS plus 0.05% Tween 20 (PBS-T) for 1 hour at 22°C. The membrane was then incubated with anti-His antibody (diluted 1:1000 in PBS-T) for 1 hour at room temperature. After 5 washes in PBS-T, the membranes were incubated for 1 hour at room temperature in PBS-T containing anti-rabbit-immunoglobulin G hors eradish peroxidase (HRP)-conjugated secondary antibody (Abcam, Tokyo, Japan). The membranes were washed 3 times in PBS-T and twice in PBS and then stained using 1 mg/mL 3,3′diaminobenzidine in PBS with 0.04% H2O2 and 8.3% methanol.
Tumor Cell Binding Assay

Binding analysis of TGFαL3-SEB to EGFR on the cancer cells was carried out by cell enzyme-linked immunosorbant assay (ELISA) assay. The 1.0 × 10⁴ A431 cells were seeded per well into 96-well plates overnight. The cells were then washed with PBS and fixed with 10% neutral formaldehyde (0.1 mol/L PBS, 10% formaldehyde, pH 7.4) at room temperature for 1 hour. After washing, the fixed cells were blocked with blocking solution (0.5% BSA in PBS) for 2 hours and incubated with different concentrations of TGFαL3-SEB and SEB (0.1, 1.0, 10, 100, and 1000 ng/μL) at 37°C for 1 hour. After being washed 5 times with PBS-T (pH 7.4), the cells were incubated with antihexahistidine HRP-conjugated antibody (1:1500) at room temperature for 1.5 hours and washed as described previously. Finally, the color was developed by tetramethylbenzidine solution with 1.5% H₂O₂, after adding stop solution (H₂SO₄) and the absorbance was read at 450 nm using a ELISA plate reader.

Splenocyte Proliferation Assay

Freshly isolated splenocytes from the spleen of healthy BALB/c mice were seeded into 96-well plates at a density of 1 × 10⁵ cells per well in the presence of 1.0, 2.0, and 6.0 μg/mL of TGFαL3-SEB and SEB, 20 μg/mL phytohemagglutinin (PHA) as a positive control and PBS as negative control. The treated cells were cultured at 37°C for 72 hours, then 50 μL sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2-H-tetrazolium (XTT), 1 mg/mL, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide was added and incubated at 37°C for 4 hours. Finally, the OD₄₅₀ was measured on an ELISA plate reader (Biorad, USA). The relative proliferating activity of the fusion protein was calculated by 100% × OD₄₅₀ of the TGFαL3-SEB fusion protein/OD₄₅₀ of the SEB.

In Vitro Tumor Cell Growth Inhibition Assay

Mouse breast cancer 4T1 cells as target cells were seeded into a 96-well plate at a density of 3.0 × 10⁴ cells/well. Following an overnight incubation period, mouse spleen, freshly separated from healthy BALB/c mice, were cocultured at an effector-to-target (E:T) cell ratio of 1:1, 10:1, 30:1, and 100:1, respectively, in triplicate to a total volume of 100 μL in the presence of 5 ng/μL TGFαL3-SEB. The plates were incubated at 37°C and 5% CO₂ for 72 hours. Tumor cell viability was determined using the XTT assay. The data were reported as the percentage of tumor cell growth inhibition (TCGİ%), which was calculated as follows:

\[
\text{TCGI%} = 1 - \frac{(A_{\text{test}} - Ab)/(Ac - Ab)}{A_{\text{control}}} \times 100,
\]

where \(A_{\text{test}}\) is the absorbance of tumor cells grown in the presence of the effector cells and Sags, \(Ab\) is the absorbance of the medium only, and \(Ac\) is the absorbance of the tumor cells grown in the medium.

Upon determination of the E–T ratio causing 50% TCGI, 4T1 cells were cocultured with freshly mice splenocytes at defined E–T ratio in the presence of various concentrations of Sags (0.05, 0.5, 5, 50, and 200 ng/μL). Cells were cultured at 37°C in 5% CO₂ for 72 hours. The remaining viable tumor cells were determined using the XTT assay. The data were reported as the TCGI% as described previously.

Statistical Analysis

All experiments were performed in triplicate and results were presented as mean ± standard deviation. Statistical comparisons were made by Student t test or analysis of variance. The accepted level of significance was \(P\) value <.05.

Results

Detection of seb-Positive S aureus Strain From Clinical Samples

To confirm the biochemical identification of \(S\) aureus isolates, a conserved region of the thermonuclease gene (\(nuc\) gene) of \(S\) aureus was amplified by PCR. The PCR amplification of the \(nuc\) gene of \(S\) aureus yielded the expected product of 318 bp (Figure 2A). Staphylococcus aureus ATCC 25923 and Staphylococcus saprophyticus ATCC 49453 were used as positive and negative controls, respectively. All the isolates were confirmed as \(S\) aureus. To detect the seb-positive \(S\) aureus strains PCR was performed using specific primers for seb gene that encodes \(S\) aureus enterotoxin type B (Figure 2B). Staphylococcus aureus ATCC 14485 and nontoxigenic \(S\) aureus strain ATCC 6538 were used as positive and negative controls, respectively. The PCR amplification of the seb gene yielded the expected product of 495 bp. The 17 seb-positive \(S\) aureus were detected among the confirmed \(S\) aureus isolates. After sequencing, we aligned their sequences and 1 seb-positive \(S\) aureus was selected as the representative of the 17 sequences for cloning purpose (data not shown).

Cloning, Expression, and Characterization of Purified of Proteins

Full-length region of mature SEB gene (seb) was amplified by PCR from seb-positive \(S\) aureus clinical isolates. As expected, yielded amplicons of 720 bp was observed on agarose gel electrophoresis (Figure 3A). The amplified product bound into similarly digested pET28a to produce pET28a:: seb recombinant vector. The cloned pET28a:: seb construct was transformed to BL21 (DE3) expression system and recombinant His-tagged SEB protein of ~28 kDa was purified by Ni-NTA column (Figure 4A) and confirmed by Western blot analysis (Figure 4B). Construction of pET28a:: tgfαL3-seb was done by inserting 100 pb synthetic DNA sequence encoding third loop of TGFαL3 and GGSGSGGG linker in 5′ of pET28a:: seb recombinant vector. This chimeric construct was transformed into E coli BL21 (DE3) for expressing TGFαL3-SEB fusion.
proteins. Protein of interest with the expected molecular weight of ~31 kDa was detected in the total cell pellets. After expression optimization (induction time, temperature, and IPTG concentration), the soluble phase in the buffer contained 80% SEB and 50% TGFαL3-SEB. The optimal TGFαL3-SEB native expression was induced with 0.5 mmol/L IPTG.
concentration at 37°C. The arrow in Figure 3B indicates the optimal native expression of TGFαL3-SEB fusion protein. Soluble TGFαL3-SEB fusion protein was purified from the supernatant of sonicated bacterial pellets through a Ni-NTA affinity column. Coomassie blue staining after SDS-PAGE separation showed that both SEB and TGFαL3-SEB were purified up to 95% homogeneity (Figure 4A). Finally, the identity of SEB and TGFαL3-SEB fusion protein was confirmed by Western blotting using the anti-His antibody (Figure 4B). The optimal purification of SEB and TGFαL3-SEB is shown in Figure 4A, lanes 1 and 4.

**TGFαL3-SEB Fusion Protein Can Bind Efficiently to EGFR-Expressing Cells**

The A431 cells were used as in vitro cell culture model to investigate the binding properties of the fusion proteins to EGFR. The A431 cells are derived from human epidermoid carcinoma with high levels of EGFR expression. After overnight culture in 96-well plates, the cells were incubated with different concentrations of 6His-tagged TGFαL3-SEB or SEB (ranging from 0.1 ng/µL to 1 µg/µL). The cells were then incubated with HRP-conjugated antihexahistidine antibody. Unlike SEB, TGFαL3-SEB fusion protein bound to A431 EGFR-expressing cells with a significant affinity (Figure 5; P < .05). The binding affinity of SEB was like the negative control, PBS.

**Third Loop of TGF-SEB Maintains Its Superantigenic Properties**

To investigate whether the fusion of TGFα-L3 to SEB can affect superantigenic activity of this protein, we compared the cell proliferation upon exposure to either TGFαL3-SEB or SEB by XTT assay. Mice splenocytes were incubated with TGFαL3-SEB or SEB or PHA at 37°C for 72 hours. As shown in Figure 6, TGFαL3-SEB maintained 95.5% of the proliferative activity of SEB at 1 µg/mL concentration of the proteins (Figure 6). We also compared fusion protein superantigenic activity in higher concentrations (2 and 6 µM/mL) of proteins to investigate whether the superantigenic activity of TGFαL3-
SEB was maintained in higher concentration of this protein. Results indicated that TGFαL3-SEB maintained 91.2% and 84.9% of the proliferative activity at 2 and 6 μg/mL TGFαL3-SEB concentrations, respectively (data not shown). As a control, PHA induced strong proliferation of splenocytes. Our results showed that fusing of TGFαL3 in N-terminal of the TGFαL3-SEB construct has no significant effects on either MHC binding or its superantigenic activity.

Third Loop of TGF-SEB Fusion Protein Can Inhibit Tumor Cell Growth In Vitro

We investigated whether TGFαL3-SEB is able to bridge between tumor cells and immune effector cells leading to the lysis of breast cancer cells. 4T1 cells, as target cells, were initially cocultured with increasing numbers of activated spleen cells as effector cells in the presence of either 5 ng/μL TGFαL3-SEB or SEB. As shown in Figure 7A, 50% inhibition of tumor cell growth by TGFαL3-SEB was achieved when the E–T ratio was around 30:1. Under this E–T ratio, SEB exerted more potent inhibitory effect than TGFαL3-SEB, whereas from the ratios of 30:1 to 300:1, the 2 curves overlapped and there was no difference between SEB and TGFαL3-SEB in TCGR5% (Figure 7A). The E–T ratio of 30:1 was used as the 50% TCGR in the subsequent treatments. The 4T1 cells were cocultured with activated spleen cells at 30:1 E–T ratio in the presence of increasing concentrations of either TGFαL3-SEB or SEB. As shown in Figure 7B, 50% inhibition of tumor cell growth by TGFαL3-SEB and SEB was achieved at 0.05 ng/μL of both proteins. Upon 5 ng/μL treatment, SEB showed much higher inhibitory effect than TGFαL3-SEB. Higher concentrations of TGFαL3-SEB showed higher cytotoxicity compared with the higher concentrations of SEB in a dose-dependent manner (Figure 7B). Also, as shown in Figure 7B, 100% inhibition of tumor cell growth by TGFαL3-SEB was achieved at 200 ng/μL of the fusion protein.

We also investigated the effect of SEB and TGFαL3-SEB fusion protein on murine splenocyte by adding 5 ng/μL proteins on increasing number of splenocytes ranging from 25 000 to 7 500 000 cells. Our results indicated that maximum proliferative effect obtained in the presence of 750 000 of murine splenocytes was 30 times more than 4T1 target cells (30:1 E–T ratio; data not shown). In higher number of splenocytes (2 500 000 and 7 500 000), the cells began to die and XTT optical density decreased presumably because of the abnormal confluency of the cells in the wells and lack of nutrients.

Discussion

T cells have multifarious antitumor effects via releasing tumor-suppressive cytokines (interferon γ [IFN-γ] and tumor necrosis factor α [TNF-α]) or cytotoxic effector molecules such as perforin,22,25-27 but generally the number of tumor-specific T cells is insufficient to mediate potent antitumor responses against progressive tumor growth.34 Superantigens can activate a large number of T cells irrespective of their antigen specificity resulting in a massive release of cytokines from T cells and monocytes. They enhance the antitumor activity of the immune system and prevent tumor growth and metastasis.7 According to our data, Sags can be used to enhance specific antigen responses. However, in vivo administration of intact Sags at sufficient therapeutic amounts could produce unwanted cytotoxic effects on normal cells. This is the main problem associated with most of the anticancer agents because of their nonspecific activities.35 Therefore, anticancer chemotherapeutics are often given at suboptimal doses, resulting in the failure of therapy, often accompanied by the development of drug resistance and tumor metastasis.18
prevents SAg from being presented to the surface of the tumor cells and subsequently prevents activation of T cells.

In 2010, Xu et al suggested that the third loop of hTGF-α (TGFα L3) retained the binding ability to EGFR. The TGFα L3 is a native ligand to its receptor, EGFR, in many human tumors. The binding of the ligand to EGFR was relatively weaker than mAbs but was sufficient enough to bring SAg toward EGFR-overexpressing tumor cells. They fused TGFα L3 to mutant staphylococcal enterotoxin A (SEA; SEAD227A, introduced by Holzer). Attempts to define the relationship between the structures of SEs and their various biologic activities have focused primarily on the activation of T lymphocytes. These studies indicated that more than 1 region of the toxin is involved in T-cell activation.

The Sag SEA binds to MHC class II molecules at 2 sites on either side of the peptide groove. Two separate but cooperative interactions to the human class II molecule human leukocyte antigen (HLA) DR1 were detected. The first high-affinity interaction to the DR1 β chain is mediated by a zinc atom coordinated to H187, H225, and D227 in SEA and H81 in the polymorphic DR1 β chain. The second low affinity site is to the DR1 β chain analogous to SEB binding and is mediated by residue F47 in SEA. Both binding sites on SEA are required for maximal activity. Thus, unlike SEB, SEA requires 2 separate binding sites for optimal activity, which may allow it to stabilize SEA interaction with TcRs as well as to activate the antigen-presenting cell by cross-linking MHC class II. SEAD227A point mutant was constructed by Holzer in order to decrease MHC class II affinity and therefore prevent unwanted binding of the Sag SEA to MHC class II molecules. This mutation (SEAD227A) resulted in an approximately 3-log reduction in affinity to HLA-DR, and consequently T-cell stimulating activity was 4- to 5-log less effective than native SEA when activation of resting T cells was assayed in terms of blast formation, expression of cell surface activation markers, and cytokine release (interleukin 2, IFN-γ, and TNF-α). Furthermore, lysis of MHC class II-positive cells was reduced approximately 1000-fold, and stimulation of T cells required 4- to 5-log higher concentrations of SEAD227A when compared to the wild-type SEA. Overall, according to their findings, the potency of the mutant SEAD227A in T-cell activation was severely reduced when compared to the wild-type Sag.

In the case of SEB, several studies have been done to detect the key fragment of SEB structure essential to T-cell proliferation. Binek et al revealed that a 17-kDa carboxy-terminal fragment of SEB was crucial for T-cell proliferation. Buelow et al indicated that residues 1 to 138 were sufficient to promote T-cell proliferation. Deletion of the first 30 amino-terminal residues of SEB eliminated activation of at least 1 VP type (V beta 3.1), while polyclonal T cells still responded to this molecule. On the other hand, analysis of site-directed mutants of SEB confirmed the importance of specific amino-terminal and central regions of SEB in MHC class II and VP interactions. In general, deletions of amino-terminal and carboxy-terminal residues of SEA and SEB reduced their stability and mitogenic activity.
The present study aimed to evaluate the antitumor activity of the recombinant TGFzL3-SEB in vitro using the third loop of TGFzL to deliver whole SEB molecule to 4T1 murin breast cancer cell line. The potency of TGFzL3-SEB was examined in a T-cell activation assay. Based on 40 independent studies, the mean percentage of EGFR expression on breast cancer cells is 45% (range 14%-91%). Since expression of EGFR is highly associated with tumor invasion and metastasis, we used the EGFR-overexpressing 4T1 breast adenocarcinoma mouse cell line41 in our in vitro study. The 4T1 mammary carcinoma cell line was originally isolated by Miller and coworkers at the Karmanos Cancer Institute.42,43 This cell line is a widely studied model for basal-like breast cancer with metastatic potential.31,44,45 We used the 4T1 breast adenocarcinoma mouse cell line as a model system because tumor growth and metastatic spread of 4T1 cells mimic very closely to human breast cancer and has the advantage of being able to be transplanted into immunocompetent recipients.46 This cell line is spontaneously capable of metastasis to several organs including lungs, liver, and brain as well as bone.31,47-49

We included controls (4T1 + media, 4T1 + SEB, and 4T1 + TGFzL3SEB) in TCGI assay to rule out direct cytotoxic effect of SEB and TGFzL3-SEB fusion proteins on 4T1 cell line. No cytolytic effect was detected when 4T1 target cells were incubated with the proteins only.

Xu et al examined the effects of TGFzL3-SEAD227A fusion protein on A431 and B16 cancer cells. The A431 cells contained as many as 2.0 × 10^6 EGF receptors per cell. The EGFR gene was indeed amplified 15- to 20-fold in A431 cell line,50 while murine melanoma B16 cells expressed lower EGFR compared to that found in A431 cells. For this reason, they used A431 cell line in their TCGI assay and found that TGFzL3-SEAD227A fusion protein inhibits the cell growth much more effectively in a dose-dependent manner compared to SEA. However, in the case of B16 cell line, SEA exerted more potent inhibitory effects than TGFzL3-SEAD227A at concentrations below 5 ng/μL. Interestingly, TGFzL3-SEAD227A showed considerable dose-dependent inhibition effect at concentrations higher than 5 ng/μL compared to SEA.24 Our results were consistent with Xu et al when 4T1 cell line was used instead of B16 cell line. Expression of EGFR in 4T1 and B16 cell lines is equally low compared to A431 cell line. In TCGI assay using 4T1 cell line, SEB showed higher inhibition effect than TGFzL3-SEB at concentrations below 5 ng/μL, but at concentrations above 5 ng/μL, TGFzL3-SEB exerted remarkable dose-dependent inhibition effect.

This evidence suggests that the ligand density is high enough at concentrations higher than 5 ng/μL TGFzL-Sag fusion protein to target Sag toward cancer cells (such as 4T1 and B16 cell lines) that express lower surface EGFR than A431. Also, as shown in Figure 7B, 100% inhibition of tumor cell growth by TGFzL3-SEB was achieved in 200 ng/μL of fusion protein, but in Xu et al study, 500 ng/μL of TGFzL3-SEAD227A caused 70% TCGI. This may be due to mutations in the SEA protein and subsequent reduction in its antiproliferative properties.

Conclusions
Our results indicated that TGFzL3-SEB fusion protein is capable of targeting EGFR-expressing cancer cells. Therefore, it may be a promising anticancer candidate for immunotherapy on EGFR-expressing tumors, but further efforts are needed to explore this potential therapeutic strategy.

Authors’ Note
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