Original Paper

The Immunogenicity and Anti-Tumor Efficacy of a Rationally Designed EGFR Vaccine

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Key Words

Cancer vaccine • EGFR • Diphtheria toxin T-domain • CpG

Abstract

Background/Aims: The abnormally activated EGFR promotes tumor growth, invasion and metastasis. Current therapeutics targeting EGFR have markedly improved the clinical outcome, but they are limited in use due to transient efficacy, frequent administration, high cost and significant toxicity. **Methods:** We rationally designed a multiepitope immunogen against EGFR, named as DEGFR^m. The immunogen is composed of an epitope peptide (EGFR²⁶⁵⁻²⁸³) and the extracellular domain III (EGFR³³⁴⁻⁵⁰⁵) of mouse EGFR. EGFR²⁶⁵⁻²⁸³ is grafted onto the translocation domain of diphtheria toxin (DTT), and EGFR³³⁴⁻⁵⁰⁵ is fused to C-terminal of DTT. Next, the immunogenicity and anti-tumor efficacies of DEGFR^m vaccine were examined in mouse tumor models. **Results:** When formulated with Alum and CpG, DEGFR^m vaccine elicits Th 1 immune responses and inhibits tumor growth in both prophylactic and therapeutic mouse tumor models. Moreover, the tumor microvasculature is markedly reduced and the tumor infiltration of CD8⁺ T lymphocytes is greatly enhanced. **Conclusions:** These data suggest that active immunization with DEGFR^m vaccine is a promising strategy for therapy of various EGFR⁺ cancers.

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Introduction

Epidermal growth factor receptor (EGFR) is a single-pass type I membrane protein and a member of receptor tyrosine kinase family. The receptor is composed of an extracellular ligand-binding domain (ECD), a helical transmembrane domain, and a cytoplasmic signaling domain. Upon binding to its ligands such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF- α), the receptor is dimerized, which activates the receptor tyrosine kinase, and in turn it initiates several signaling cascades such as RAS-RAF-MEK-ERK, phosphatidylinositol-3 kinase-Akt, PLCgamma-PKC etc, which contribute to cell proliferation, angiogenesis, and apoptosis resistance [1, 2].

The abnormal activation of EGFR by aberrant gene amplification or mutation has been associated with tumor progression and poor survival in a number of human cancers including head and neck, colon, lung, ovarian, prostate and pancreatic cancers [3-8]. To date, several anti-EGFR agents have been developed for cancer therapy. The clinically approved therapeutics included small molecular inhibitors such as Iressa and Tarceva, and monoclonal antibodies (mAbs) such as Cetuximab and Panitumumab [2]. However, the clinical application of small molecular inhibitors are limited by their biocompatibility, pharmacokinetics and stability [9], and the monoclonal antibodies are limited in clinical use due to high cost, infusion reactions, toxicity, and emergence of resistance [10, 11]. It is therefore an urgent need to develop alternative strategies for safer, less toxic, low cost and more efficacious therapeutics.

Active immunization with tumor specific antigens or epitopes formulated with strong adjuvants such as complete Freund's adjuvant (CFA) has been shown therapeutic effect on a number of cancer types [12-14]. The ECD of mouse EGFR is responsible for ligand binding and the receptor dimerization, and is composed of four structural sub-domains, among which the sub-domain II (aa190-334) is responsible for the receptor dimerization, and the sub-domain III (aa335-506) is responsible for the ligand-binding [15]. The sub-domain III possesses two partially overlapping epitopes recognized respectively by anti-tumor monoclonal antibodies Cetuximab and Panitumab. Cetuximab has been clinically approved for treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer [2]. Panitumumab has been clinically approved to treat metastatic colon cancer [2]. In this study, we investigated whether active immunization with domains II and III could generate effective immune responses to ablate EGFR-positive tumor cells when immune self-tolerance is breached.

In our previous studies, we show that the translocation domain of diphtheria toxin (DTT) is a safe antigen carrier capable of breaking immune self-tolerance by virtue of its universal help T cell epitopes [16, 17]. Moreover, DTT-based antigen is immunogenic when formulated with Alum, a widely used adjuvant in human vaccination. We and others also show that CpG enhances antitumor immunity when formulated with cancer vaccine [18, 19]. Based on these observations, we designed a vaccine formulation comprising a recombinant immunogen against EGFR, named as DEGFR^m vaccine, which was composed of DEGFR^m CpG and Alum adjuvants. We assessed the immunogenicity and anti-tumor efficacies of DEGFR^m vaccine in two mouse tumor models, in which mice are administrated with Lewis lung tumor cells and 4T1 breast tumor cells.

Materials and Methods

Peptides, cell lines, mice, and adjuvants

4T1 cells, and Lewis cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI 1640 with 10% fetal bovine serum (FBS). Female C57BL/6 and Balb/c mice (6-8 weeks old) were purchased from Chinese Academy of Sciences, Shanghai Experimental Animal Center, and housed on a 12-h light/dark cycle under specific pathogen-free conditions with food and water ad libitum. All animal studies were performed in accordance with the guidelines approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. Aluminum hydroxide gel (Alum) was purchased from Invitrogen (Carlsbad, CA, USA). A mouse-specific CpG oligo deoxynucleotide (CpG ODN1826, TCCATGACGTTTCCTGACGTT) and M-EGFR^p peptide (AMGKLL SLIKGVIVHRLEGVE-GPSL-DTCPPLMLYNPTT YQMDVNPEGKYSGATCV) were synthesized by GeneScript (Nanjing, China). All chemical reagents were of analytical grade.

The epitope choice and molecular design

We analyzed the structure of complex EGFR-CETUXMAB which was available (PDB code 1YY9). All interatomic distances between residues belonging to the cytokine and residues belonging to the receptor were computed with Discovery Studio 3.5. In order to insert the mouse EGFR epitopes in the DTT domain

scaffolds, our lab had found one site that can be used for the most of the foreign epitope for eliciting antibody responses. By employing comparative modeling we have designed our protein in which epitope derived from mouse EGFR²⁶²⁻²⁹² epitope, has been inserted into DTT (T domain of diphtheria toxin). Finally, the amino acids 88-94 of DTT was replaced by EGFR²⁶⁵⁻²⁸³ (NH₂-PLMLYNPTTYQMDVNPEG-COOH) using Discovery studio 3.5. Homology model of fusion protein was built based on the X-ray structure of DTT (PDB entry 1MDT, 2.3Å) using Modeller9V7 [16].

Expression vectors

The DTT expression vector was described previously [16]. The recombinant DNA fragment encoding DTT¹⁻⁸⁷-EGFR²⁶⁵⁻²⁸³-DTT⁹⁵⁻¹⁷⁶-EGFR³³⁴⁻⁵⁰⁵ was chemically synthesized, cloned into pET-32a after BamHI and Xho I digestion.

DEGFR^m expression and purification

The expression vector was transformed into E. coli Origami B (DE3). A single colony was inoculated into 500 ml of LB media and cultured on a shaker at 37°C until OD_{600nm} reached to 0.6. Then, IPTG (isopropylthiogalactoside) was added to a final concentration of 0.5 mM. The culture was further incubated at 28°C for 16h, after which cells were collected by centrifugation, lysed by ultrasonication, and the protein was purified by Ni-NTA affinity chromatography. The purified protein was digested with PSP (PreScission Protease) in 50 mM Tris-HCl, 150 mM NaCl, 1mM EDTA, pH 7.0, at 4°C for 12h, and the cleaved his tag was removed by Ni-NTA affinity column. The flow-through was concentrated and buffer exchanged into PBS buffer. The protein purity was analyzed by SDS-PAGE.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA and cDNA from tumor cell were got by described preciously. PCR was performed with KOD plus polymerase (Toyobo, Japan). The primers for detecting mEGFR are CGCCGCAAAGTTTGTAAATGGCATAGGC and CCGCTAGA CGTGGTTTCACGGCCTTGCAGTCTTTC. The condition for PCR reaction is 94°C (2 min), 94°C (1 min), 55°C (30 s), 68°C (2 min), 30 cycle, 68°C (10 min).

Mice immunizations

The vaccine was prepared by mixing 50 µg of DEGFR^m and 0.3 mg Alum, with or without 30 µg CpG, in 0.1 ml PBS (phosphate buffer saline) buffer, named as DEGFR^m+Alum+CpG and DEGFR^m+Alum, respectively. Mice were intramuscularly immunized four times at two-week interval. In the control experiments, mice received either PBS or DTT+Alum+CpG. Sera were collected from the mice 7 days after each immunization.

Antibody detection

Anti-EGFR antibody in mouse sera was detected by ELISA. A microtiter plate was coated with 1µg/mL mouse EGFR (Sino Biological Inc) in 100µg PBS in each well, and incubated at 4°C overnight. The nonspecific binding sites were blocked with 5% FBS + 0.05% Tween 20 in PBS. 100µg serum diluted 100-fold was added, and incubated at 37°C for 2 h. After the plate was washed three times with PBS, HRP-conjugated goat anti-mouse IgG antibody (200ng/ml, Santa Cruz Biotechnology) was added and incubated at 37°C for 1h. Then the plate was washed three times with PBS, and incubated with 100µg TMB solution (Beyotime, cat num P0209) for 15 min, and the reaction was stopped by adding 25µl/well 2M H₂SO₄. The OD₄₅₀ was measured by a microplate reader (PE EnSpire™).

Western blot analysis

Ten ng purified DEGFR^m proteins was loaded on 12% SDS-PAGE, and transferred to PVDF membrane. The membrane was incubated with 5% milk-PBS at room temperature for 1h, then 1µg/ml anti-EGFR antibody (R&D systems) in 2% milk-TBS at 4°C overnight. After washing three times with PBS, the membrane was incubated with FITC-conjugated goat anti-mouse IgG (2ng/ml) at room temperature for 2h, washed three times with PBS, and analyzed by Odyssey fluorescence imaging system (LICOR, US).

Mouse tumor model experiments

For the 4T1 tumor model experiments, Balb/c mice (n=3 per group) were immunized with indicated vaccine, and on day 32 after the primary immunization, mice received 2 × 10⁵ 4T1 cells. Tumor sizes were

measured by calipers, and calculated using equation: volume = [(length) × (width)²]/2. On day 22 after mice received tumor cells, the mice were sacrificed, and tumors were collected for weight measurement.

For the Lewis tumor model of preventive setting, C57BL/6 mice (n=5 per group) were immunized with indicated vaccine, and on day 21 after the primary immunization, mice received 2×10^5 Lewis cells. Tumor sizes were measured as mentioned above. On day 22 after mice received tumor cells, mice were sacrificed, and tumors were collected for weight measurement and immunohistochemistry analysis.

For the Lewis tumor model of therapeutic setting, C57BL/6 mice (n=5 per group) were immunized with indicated vaccine on day 7 after mice received 2×10^5 Lewis cells. Tumor sizes were measured as mentioned above. On day 23 after mice received tumor cells, the mice were sacrificed, and tumors were collected for weight measurement.

T cell proliferation

Splenocytes were isolated from C57BL/6 mouse by Ficoll-Paque density gradient centrifugation on day 21 after primary immunization, seeded into a 96-well plate at 1×10^5 cells/well in 100 µl of RPMI-1640 +10% FBS, and incubated for indicated days at 37°C for 4h. After stimulation with 10 µg/ml mouse EGFR (Sino Biological Inc) for 72 h, 10 µl/well MTT solution (Beyotime, Jiangsu, China) was added, and incubated at 37°C for 4h, and then 100 µl/well dimethylsulfoxide (DMSO) was added. The plates were kept on an orbital shaker for 5 min. The absorbance at 570 nm was measured by a microplate reader (PE EnSpire™) [19, 20].

For T cell subtype detection, freshly isolated splenocytes were stimulated with mouse EGFR (Sino Biological Inc) for 72 h. After washing 3 times with PBS, cells were stained with anti-CD3-PE, anti-CD4-FITC and anti-CD8-APC antibodies (BD Biosciences, USA) in $1 \mu\text{g}/10^6$ cells in 100µl PBS with 2% FBS, and analyzed using BD FACSCalibur™ (Becton Dickinson, USA). The unstimulated cells were used as a negative control. All experiments were repeated 3 times.

Detection of intracellular interferon-γ (IFN-γ)

Intracellular IFN-γ was examined as described [19]. Briefly, splenocytes were incubated with brefeldin A (10mM, MultiSciences, China) for 6 hours to block IFN-γ secretion. Then the cells were stained with anti-CD3-FITC and anti-CD8-PE antibodies ($1\mu\text{g}/10^6$ cell, eBiosciences) in 100µl 2% FBS-PBS. After washing with PBS, cells were fixed with IC fixation buffer (MultiSciences) at room temperature for half an hour, permeabilized with $1 \times \text{EB}$ permeabilization buffer, stained with anti-mouse IFNγ-APC ($1\mu\text{g}/10^6$ cell, eBioscience), and analyzed by FACS Calibur™ (Becton Dickinson, USA).

Quantitative PCR assay

Tumor tissue RNA was prepared using TRIzol reagent (Invitrogen). 1 µg total RNA was reverse-transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Fermentas, USA) following manufacturer's instructions. Real time PCR was performed using the SYBR Green kit (Fermentas, USA) following the manufacturer's instructions. Data was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method [19, 21]. The primer sequences are listed in Table 1.

Immunohistochemistry analysis

The formalin-fixed, paraffin-embedded tumor tissues were cut into 4µm-slides according to Foy KC et al. [22]. The slides were blocked with 5% FBS in PBS at room temperature for 2h, and incubated at 37°C for 2h with anti-mouse CD8 and CD3 antibodies. After washing 3 times with PBS, the slides were further incubated with HRP-labeled secondary antibody for 1h. The amount of bound secondary antibody was visualized with DAB solution (3, 30-diaminobenzidine tetrahydrochloride) and the slides were counterstained with hematoxylin, and evaluated by Image J software on five representative fields by two investigators.

Table 1. Primers used in qPCR

Primer	Sequence 5'-3'
mGAPDH-F	GCTAGGACTGGATA AGCAGGG
mGAPDH-R	AATCCGTTACACCGACCTT
mCD8a-F	TCAGTCTCTGCTGCC AGTC
mCD8a-R	TCACAGGCGAAGTCCAATCC
mIL-2Rα-F	AACACCACCGATTCTGGCT
mIL-2Rα-R	GCTGGCCACTGCTACCTTAT
mIFN-γRβ-F	TCCTCGCCAGACTCGTTTTC
mIFN-γRβ-R	GGGTCATTGCTGGAAGGTGA
mEGFR-F	CGCCGAAAGTTTGTAATGGCATAGGC
mEGFR-R	CCGCTAGACGTGGTTCACGCCCTTCAGTCTTTC

Cytotoxicity assay

Lymphocytes (effector cells) were isolated from immunized mouse spleen, co-cultured with Lewis cells (target cells) at the effector to target ratio of 50:1, 20:1, and 10:1, with total cell concentrations adjusted to 2×10^5 cells/ml in 96-well plate. After co-culture for 20h, 10 μ l MTT solution was added, and the culture was further incubated at 37°C for 4h. Then, 100 μ l DMSO was added, and the plate was placed on an orbital shaker for 5 min. The OD_{570nm} value was measured by a microplate reader mentioned above, and the cell killing activity was calculated using equation: killing ratio (%) = $[1 - (\text{co-culture OD value} - \text{effector cells alone OD value}) / \text{target cell alone OD value}] \times 100\%$ [22].

Anti-EGFR PAb inhibit EGFR activation

Lewis cells were cultured in 6-well plates (Costar), and incubated with serum-free medium (DMEM) overnight. Then anti-EGFR antibody purified from DEGRF^m immunized mouse sera was added to the wells to final concentration of 0.2 mg/ml. The cells were incubated at 37°C, for 2 h. Next, the cells were incubated with mice EGF (100 ng/ml) for 10 min in DMEM media. The cells were lysed in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with 50 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA and 1 mM phenylmethylsulphonylfluoride. Cell extracts were loaded to 8% SDS-PAGE gels and transferred to PVDF membranes. The membrane was incubated with 5% milk-PBS at room temperature for 1h, then with anti-EGFR antibody in 2% milk-TBS at 4°C overnight. After washing three times with PBS, the membrane was incubated with FITC-conjugated goat anti-mouse IgG (2ng/ml) at room temperature for 2h, washed three times with PBS, and analyzed by Odyssey fluorescence imaging system (LICOR, US) [23,24].

Statistical analyses

Statistical significance was analyzed by GraphPad Software, and multiple comparisons were performed using one-way ANOVA. *indicates $P < 0.05$, **indicates $P < 0.01$, and ***indicates $P < 0.001$, and ns indicates no significant difference.

Results

The rational design of the immunogen DEGRF^m

Previous studies have shown that DTT can help break immune self-tolerance when an immunogen is fused to its C-terminal [16]. For immunogen specific against mEGFR, both ECD domain II (aa190-334) and domain III (aa335-505) are considered as they are functionally important for the receptor activation. However, Domain II is difficult to be solubly expressed in *E. coli* because of its high cysteine content [24]. Interestingly, Lei Zhu found that a B cell epitope peptide in the β -hairpin loop (aa 262-292) directly involving in the receptor dimerization could induce high titers of anti-EGFR antibody and anti-tumor effect when formulated with CFA [25]. Therefore, we chose this peptide as one part of the immunogen. In order to mimic the native conformation of the epitope peptide, we grafted the peptide onto the DTT at positions aa⁸⁸⁻⁹⁴ that has been shown an ideal site for epitope presentation [16]. EGFR²⁶⁵⁻²⁸³ is grafted to minimize structural perturbation of the carrier DTT, according to the method of Li Zhang et al. [16]. To achieve higher anti-tumor efficacy of the vaccine, we considered fusion of ECD subdomain III to the C-terminal of DEGRF^p. The subdomain III contains two partially overlapping epitopes recognized by monoclonal antibody Cetuximab and Panitumumab, respectively. The resulting immunogen named DEGRF^m. We examined the immunogenicity of the resulting immunogens in mice, with EGFR²⁶⁵⁻²⁸³ fused to C-terminal of DTT (D-EGFR^p) or MVF (M-EGFR^p) as controls (Fig 1). As shown in Fig. 1A, DEGRF^p elicits stronger antibody response against mouse EGFR than D-EGFR^p suggesting that a more stable conformation of the epitope peptide is beneficial to eliciting antibody response. No anti-EGFR antibody response was detectable in M-EGFR^p immunized mice, probably due to adjuvant Alum, which is milder than CFA used in the previous M-EGFR^p study [25].

To achieve higher anti-tumor efficacy of the vaccine, we considered fusion of ECD subdomain III to the C-terminal of DEGRF^p. The subdomain III contains two partially overlapping epitopes recognized by monoclonal antibody Cetuximab and Panitumumab,

Fig. 1. Immunogenicity of purified recombinant immunogens against EGFR. **A.** Female C57BL/6 mice (6-8 weeks old, n=5) were immunized on day 0, day 14 and day 28 with 50µg indicated immunogen and 0.3 mg Alum. After 3 immunizations, anti-EGFR antibody responses were measured by ELISA. DEGR^P: graft the peptide (EGFR²⁶⁵⁻²⁸³) onto the DTT at positions aa88-94. D-EGFR^P: the peptide (EGFR²⁶⁵⁻²⁸³) fused to C-terminal of DTT; M-EGFR^P: the peptide (EGFR²⁶⁵⁻²⁸³) fused to C-terminal of MVP. **B.** SDS-PAGE analysis of purified DTT and DEGR^m. DEGR^m: fusion of ECD subdomain III to the C-terminal of DEGR^P. **C.** Western blot analysis of DEGR^m with EGFR-specific antibody. DTT was used as a control. **D.** Female C57BL/6 mice (6-8 weeks old, n=7) were immunized on day 0, day 14 and day 28 with 50µg indicated immunogen and 0.3 mg Alum. After 3 immunizations, anti-EGFR antibody responses were measured by ELISA. DE-EGFR^m: fusion of ECD subdomain III to the C-terminal of DEGR^P. DEGR^w: fusion of ECD subdomain III to the C-terminal of D-EGFR^P. **E.** Female C57BL/6 mice (6-8 weeks old, n=3) were immunized on day 0, day 14 and day 28 with 50µg indicated immunogen and 0.3 mg Alum, with or without 30µg CpG. After 3 immunizations, splenocytes were isolated and stimulated with 10 µg/ml mouse EGFR for 72 h. Then the proliferation of the splenocytes was measured by MTT assay.

respectively. The resulting immunogen was named DEGR^m. We found that the recombinant protein DEGR^m exists in both soluble and insoluble form when expressed in E. coli. The soluble fraction was purified to homogeneity as shown in Fig. 1B. Western blot analysis confirmed that DEGR^m is recognized by an EGFR specific antibody (Fig. 1C). The final yield of the purified protein is 0.5mg/L.

We next examined the anti-tumor immunity of DEGR^m in mice using DTT and DEGR^w as control. DEGR^w is composing of DTT C-terminal fused with EGFR³³⁴⁻⁵⁰⁵, and then fused again with EGFR²⁶²⁻²⁹² as C-terminal. Fig. 1D shows that both DEGR^m and DEGR^w induced robust anti-EGFR antibody responses with DEGR^m treated mice had higher antibody titers.

To examine whether the vaccination elicit cellular memory responses, we *in vitro* stimulated splenocytes from the immunized mice of various groups with DEGR^m, and examined the cell proliferation by MTT assay. As shown in Fig. 1E, significant cell proliferation was detected in the splenocytes from mice immunized with DEGR^m+Alum and DEGR^m+Alum+CpG, but not in those immunized with DTT+Alum+CpG, DTT+Alum, or PBS.

DEGR^m inhibits tumor growth and neoangiogenesis

To examine whether the vaccination inhibits tumor growth, we measured the tumor volumes after the immunized mice received the breast cancer 4T1 cells or Lewis lung cancer cells. Fig. 2A shows that, 4T1 tumor grew significantly slower in mice immunized with DEGR^m+Alum+CpG while the tumor growth rate was similar among mice immunized with PBS, or DTT +Alum+CpG, or DEGR^m+Alum. On day 22 after the mice were challenged with the tumor cells, tumor weights were measured. Consistent with the volume measurements, tumor weights in DEGR^m+Alum+CpG treated mice were reduced as compared with those in PBS, or DTT +Alum+CpG, or DEGR^m+Alum.

Fig. 2B shows that DEGR^m+Alum+CpG vaccination also inhibits the growth of Lewis lung tumor in mice. Furthermore, tumors from DEGR^m+Alum+CpG treated mice show significantly reduced microvascular density (Fig. 2E-F), suggesting that active immunization with DEGR^m inhibits neoangiogenesis.

Fig. 2. Anti-tumor efficacy of DEGFR^m in mouse tumor models. **A.** Preventive breast cancer model. Balb/c mice (n=3 per group) were immunized with 50μg DEGFR^m and 0.3 mg Alum, with or without 30μ CpG, and on day 32 after the primary immunization, mice received 2 × 10⁵ 4T1 cells. Tumor sizes were measured by calipers, and calculated using equation: volume = [(length) × (width)²]/2. On day 22 after mice received tumor cells, the mice were sacrificed, and tumors were collected for weight measurement. **a.** Immunization schedule. **b.** 4T1 tumor growth curve. **c.** tumor weight. **B.** Preventive lung cancer model. C57BL/6 mice (n=5 per group) were immunized with 50μg DEGFR^m and 0.3 mg Alum, with 30μ CpG, and on day 21 after the primary immunization, mice received 2 × 10⁵ Lewis cells. Tumor sizes were measured as mentioned above. On day 22 after mice received tumor cells, mice were sacrificed, and tumors were collected for weight measurement. **a.** Immunization schedule. **b.** tumor growth curve. **c.** tumor weight. **D.** Tumor samples from mice in B. **E.** and **F.** Tumor vasculature analysis from mice in B. The blood vessels were stained with anti-CD31 antibody and the vasculature densities were measured using Image J software.

measurement and immunohistochemistry analysis. **a.** Immunization schedule. **b.** tumor growth curve. **c.** tumor weight. **C.** Therapeutic lung cancer model. C57BL/6 mice (n=5 per group) were immunized with 50μg DEGFR^m and 0.3 mg Alum, with 30μ CpG, on day 7 after mice received 2 × 10⁵ Lewis cells. Tumor sizes were measured as mentioned above. On day 23 after mice received tumor cells, the mice were sacrificed, and tumors were collected for weight measurement. **a.** Immunization schedule. **b.** tumor growth curve. **c.** tumor weight. **D.** Tumor samples from mice in B. **E.** and **F.** Tumor vasculature analysis from mice in B. The blood vessels were stained with anti-CD31 antibody and the vasculature densities were measured using Image J software.

To examine whether the DEGFR^m+Alum+CpG vaccination has any therapeutic effect on mouse tumors, we immunized mice 7 days after the mice received Lewis tumor cells, and measured the tumor volumes thereafter. As shown in Fig. 2C, tumors grew significantly slower in mice treated with DEGFR^m+Alum+CpG than those in control mice treated with PBS or DTT+Alum+CpG. Consistently, tumor weights on day 23 after the tumor cell challenge were significantly smaller in DEGFR^m+Alum+CpG treated mice. These results indicate that DEGFR^m vaccine has a significant therapeutic effect on mice challenged with Lewis tumor cells. Taken together, these results indicate that DEGFR vaccine have a significant tumor inhibition effect.

DEGFR^m vaccine induces Th1-type of immunity

It has been shown a Th1-type of immune response is required for efficient anti-tumor immunity. Therefore, we tested whether DEGFR^m elicits Th1-type of immune response. Fig. 3A shows that DEGFR^m vaccination induced a strong mEGFR specific antibody responses. The

Fig. 3. DEGRF^m elicits Th1 type of immunity. Female Balb/c mice (n=5 per group) were immunized on day 0, day 14 and day 28 with 50μg DEGRF^m and 0.3 mg Alum, with or without CpG. A. After 3 immunizations, anti-EGFR antibodies were measured by ELISA. B. Splenocytes (effector cell) were isolated from immunized mouse spleens, co-cultured with Lewis cells (target cells) at the effector to target ratio of 50:1, 20:1, and 10:1. Cells were co-cultured for 5 h at 37°C. 10μl MTT solution was then added, and the culture was further incubated at 37°C for 4h. Then, 100 uL DMSO was added, The OD570nm value was measured by a microplate reader. The cell killing activity was calculated using equation: killing ratio (%) = [1 - (co-culture OD value - effector cells alone OD value)/target cell alone OD value] × 100%. C. T cell population analysis. The splenocytes from DEGRF^m+Alum+CpG group were stained with anti-CD3-PE, anti-CD4-FITC and anti-CD8-APC antibodies, and analyzed using BD FACS Calibur™. D. INF-γ⁺CD8⁺T cell analysis. Splenocytes from DEGRF^m+Alum+CpG group were incubated with brefeldin A for 6 hours to block IFN-γ secretion. Then the cells were stained with anti-CD3-FITC and anti-CD8-PE antibodies. After washing with PBS, cells were fixed with IC fixation buffer, permeabilized with 1 × EB permeabilization buffer, stained with anti-mouse IFNγ-APC, and analyzed by FACS Calibur™).

IgG antibody level was higher when the vaccine was formulated with CpG. No anti-mEGFR antibody was detected in DTT or PBS treated mice. Moreover, DEGRF^m induced significant levels of IgG2a and 2b, and the level increased further in the presence of CpG, suggesting that a Th1-type of immune response is induced, and is consistent with previous reports, CpG help to induce stronger type 1 immunity.

To examine whether the vaccine elicit cytotoxic T-lymphocyte responses, an essential component of Th1 type immunity, Lewis tumor cells were co-cultured with the lymphocytes from mice immunized with DEGRF^m vaccine. As shown in Fig. 3B, the lymphocytes from mice immunized with DEGRF^m+Alum or DEGRF^m +Alum+CpG have higher levels of cytotoxicity than mice treated with PBS or DTT +Alum.

We further measured the levels of CD8⁺ T cells population and IFN-γ secreting effector cells. As shown in Fig. 3C, CD8⁺T cells population was increased by 2.5 fold in DEGRF^m+Alum+CpG group as compared to those of the control groups. The IFN-γ positive CD8⁺ T cells were increased by 2 fold. Taken together, our data show that DEGRF^m+Alum+CpG elicited Th1 type of immune response.

DEGRF^m vaccination enhanced tumor infiltration of CD8⁺T cell

Lymphocyte infiltrating into tumors is a prognostic factor in many cancer treatments [26]. Fig. 4A and B show that the level of tumor infiltrating CD8⁺ T cells is significantly increased in mice treated with DEGRF^m+Alum+CpG as compared with other groups of mice. Quantitative reverse transcription-PCR(qRT-PCR) also demonstrated increased expression of CD8a, IFN-γRβ and IL-2Rα in mice vaccinated with the DEGRF^m+Alum+CpG as compared to other groups of mice (Fig. 4C-E), which indicate increased CD8⁺ T cells and Th1-type of immune cells in tumors of mice treated with DEGRF^m +Alum+CpG.

Fig. 4. Tumor infiltration of CD8⁺ T cells and Th1 cytokines in tumor issue. Female C57BL/6 mice (6-8 weeks old, n=5) were immunized on day 0, day 14 and day 28 with 50μg DEGRF^m and 0.3 mg Alum, with CpG. On day 21 after the primary immunization, mice received 2×10^5 Lewis cells. On day 22 after mice received tumor cells, mice were sacrificed, and tumors were collected. A. Immunohistochemical staining of tumor tissue with anti-CD8 antibody. B. CD8⁺ T cell density measurements by Image J software (National Institutes of Health). C-E. Tumor tissue RNA was extracted and analyzed for expression of IFNγRβ, IL-2Rα and CD8a by quantitative reverse transcription-PCR (qRT-PCR).

Anti-EGFR antibody inhibit EGFR activation

Anti-EGFR antibody can inhibit tumor cells proliferation by blocking EGFR and downstream signaling transduction. Sera from immunized mice were assayed. Lewis cells were incubated with anti-EGFR antibody purified from DEGRF^m immunized mice or unimmunized mice. Anti-EGFR antibody can inhibited EGFR phosphorylation by inhibition of exogenous EGF binding (Fig. 5). But without addition of exogenous EGF, there is no obvious inhibition.

Discussion

The extracellular domain (ECD) of EGFR has been successfully targeted for the development of inhibitory antibodies, in particular the subdomain III of ECD [27, 28]. In this study, we designed a multi-epitope vaccine against two separate ECD regions of mouse EGFR. The results showed that the vaccine enhanced Th1 type of immune responses with high titers of IgG and a significant level of cytotoxic T lymphocytes. In addition, it could elicit humoral and cellular immune response reduces tumors growth against two separate ECD regions of not only in prophylactic setting but also in therapeutic setting.

It is well appreciated that a Th1 type of immune response is required for anti-cancer immunity. As a clinically approved adjuvant for human vaccine, alum is rarely used in formulation of human cancer vaccine because it facilitates Th2 type of immune response. Interestingly, work from Lu et al. show that in presence of CpG, alum formulated cancer vaccine is effective in eliciting Th1 type of immune responses [19]. Consistent with this report, we showed that CpG and Alum formulated DEGRF^m could elicit Th1 type of immune response and anti-tumor effect in mouse tumor models. This more balanced immune response is assumed to be more beneficial for eliciting systemic anti-tumor immune response.

Fig. 5. Anti-EGFR antibody inhibit EGFR phosphorylation. Lewis cells were growth in starvation condition overnight and then incubated with anti-EGFR antibody. EGFR phosphorylation was measured by western blot. Photograph shows EGFR phosphorylation in Lewis cells stimulated with EGF in 0.2mg/mL anti-EGFR antibody get from DEGRF^m immunized mice.

DTT has been shown as a safe vaccine carrier and epitope peptide scaffold, and can help to break immune self-tolerance [16]. When a TNF epitope grafted onto DTT, it maintained in a native like conformation. Our data show an EGFR epitope grafted onto DTT at the similar position also induces antibody response against native EGFR, which demonstrates again that DTT is a safe and effective carrier for cancer vaccine design for targeting self-molecules in particular.

Previous studies on EGFR vaccines, eukaryo expressing system were employed to generate recombinant protein immunogen because of their large molecular size and cysteine rich property that exclude the soluble expression in E coli [24, 29, 30]. DEGFR^m circumvents the eukaryotic expression system which substantially reduces the manufacture cost. Therefore, a low cost vaccine is manufactory feasible with our design strategy. By including CpG in our vaccine formulation, Alum-induced Th2 type of immune response is suppressed and Th1-type of immune response is enhanced. This more balanced immune response is assumed to be more beneficial for eliciting systemic anti-tumor immune response.

Conclusion

Data from this study demonstrate that administration of vaccine composed of DEGFR^m, CpG and alum could generate both B cell and T cell responses associated with its anti-tumor activity in mice. Our design strategy warrants to investigate the clinical benefits and therapeutic potential against EGFR over-expression tumors.

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Disclosure Statement

The authors indicate no potential conflicts of interest.

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