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Immunogenicity of a xenogeneic multi-epitope HER2$^+$ breast cancer DNA vaccine targeting the dendritic cell restricted antigen-uptake receptor DEC205

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ABSTRACT

Breast cancer was ranked first in global cancer incidence in 2020, and HER2 overexpression in breast cancer accounts for 20–30% of breast cancer patients. Current therapeutic strategies increase the survival rate, but resistance to them occurs frequently, and there is an urgent need to develop novel treatments such as DNA vaccines which can induce a specific and long-lasting immune response against HER2 antigens. To enhance the immunogenicity of DNA vaccines, dendritic cells (DCs) can be targeted using multi-epitope proteins that provide accurate immune focusing. For this purpose, we generated a DNA vaccine encoding a fusion protein composed of 1) *in silico* discovered antigenic epitopes of human and rat HER2 proteins (MeHer2) and 2) a single-chain antibody fragment (ScFv) specific for the DC-restricted antigen-uptake receptor DEC205 (ScFvDEC). The xenogeneic multi-epitope DNA vaccine (pMeHer2) encodes three only T-cell epitopes, two only B-cell epitopes, and two T and B cell epitopes, and pScFvDEC-MeHer2 vaccine additionally encodes ScFvDEC introduced at the N terminus of the MeHer2. Then, mouse groups were immunized with pScFvDEC-MeHer2, pMeHer2, pScFvDEC, pEmpty, and PBS to determine the elicited immune response. pScFvDEC-MeHer2 vaccinated mice showed a strong IgG response ($P < 0.0001$) and pScFvDEC-MeHer2 induced a significant IgG2a increase ($P < 0.01$). The percentages of both IFN-γ secreting CD4 and CD8 T cells were higher in mice immunized with pScFvDEC-MeHer2 compared with the pMeHer2. pScFvDEC-MeHer2 and pMeHer2 secreted significantly higher levels of extracellular IFN-γ compared with to control groups ($P < 0.0001$). In addition, the IFN-γ level of the pScFvDEC-MeHer2 vaccine group was approximately two times higher than the pMeHer2 group ($P < 0.0001$). Overall, this study identified the pScFvDECMeHer2 construct as a potential DNA vaccine candidate, supporting further studies to be conducted on HER2$^+$ animal models.

1. Introduction

Breast cancer ranks first in global cancer incidence in 2020 and is the leading cause of cancer death among women worldwide, representing 15.5% of cancer mortality. This disease is an important public health problem among women considering that it accounts for one in four cancer cases and one in six cancer deaths

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[1]. Breast cancer, a heterogeneous disease, is classified according to the molecular characteristics based on estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expressions [2]. The molecular types of breast cancers are luminal A (ER/PR+, HER2−, KI67−), luminal B (ER/PR +, HER2−/+, KI67+), HER2 positive (ER/PR−, HER2+) and triple negative (ER/PR−, HER2−) with the prevalence 23.7%, 38.8% / 14%, %11.2, and 12.3% of each subtype, respectively [3].

HER2 is a transmembrane tyrosine kinase receptor and belongs to the epidermal growth factor (EGF) receptor family that mediates cell proliferation, differentiation, and survival [4]. The HER2 receptors are overexpressed in colorectal, ovarian, pancreatic, and prostate cancers as well as in invasive breast cancer [5]. The overexpressed HER2 is detected in about 20–30% of breast cancer patients and has been linked to a more aggressive disease, poor response to traditional treatment, metastasis of the cancer, and poor prognosis [6].

To date, major advances have been achieved in the therapy of HER2 positive breast cancer by the help of surgery, radiotherapy, chemotherapy, and targeted therapy including monoclonal antibodies, antibody drug conjugates, and small molecule inhibitors. However, these therapies have disadvantages, such as metastases cannot be prevented with surgery, radiotherapy, or chemotherapy, and many patients have developed resistance to targeted therapy and chemotherapy [7–9]. The limited success of current therapies underlines the need for the development of an effective vaccine to increase survival rates in breast cancer patients.

Vaccines are promising, cost-effective approaches for the prevention, treatment, and eradication of cancer. In addition, they can provide long-term immune memory, which is critical to prevent tumor recurrence [10]. HER2 is the most popular antigen for the development of $HER2^+$ breast cancer vaccines. Several types of $HER2^+$ breast cancer vaccines, such as protein vaccines [11–13], peptide vaccines [14–17], dendritic cell-based vaccines [18–20], and DNA vaccines [21,22] have been extensively tested in clinical trials. Despite the encouraging results that have been observed in clinical trials, further refinement of the DNA vaccine strategy is required [23,24]. The efficiency of DNA vaccines can be improved by using two different strategies. The first is to increase their immunogenicity through *in silico* design of antigens encoded by DNA vaccines. For example, the immune protection of conventional DNA vaccines based on the integral HER2 gene is imperfect because they contain protein fragments other than specific T and B cell epitopes [25–27]. For this reason, a multiple B and T cell multi-epitope DNA vaccine strategy was adopted in the present study to confer accurate immune focusing [28–30]. The second strategy is to increase antigen presentation. There is an endocytic receptor on DCs called DEC205 (CD205), which has an antigen presentation function and has been widely used in vaccine development studies. Targeting DCs via the DEC205 surface receptor may increase antigen presentation to major histocompatibility complex I (MHC I) and major histocompatibility complex II (MHC II) molecules. The efficiency of DNA vaccines has been shown to be increased by a single chain variable fragment (ScFv) of an antibody that targets DEC205 [31–34].

In this study, we generated a novel DNA vaccine (pScFvDEC-MeHer2) encoding a fusion protein comprised of the xenogeneic HER2 multi-epitope antigen (MeHer2) and ScFvDEC to increase immunogenicity. After *in vitro* characterization of the constructed pScFvDEC-MeHer2 and investigating its ability to express the protein in HEK 293T cells, BALB/c mice were immunized with pScFvDEC-MeHer2 intramuscularly to determine *in vivo* humoral and cellular immune responses. Our results suggest that using HER2 xenogeneic multi-epitope targeted to DCs for the development of breast cancer DNA vaccines may open new doors for the treatment of $HER2^+$ breast cancer.

2. Materials and methods

2.1. Predicted of xenogeneic vaccine epitopes

The xenogeneic multi-epitope used in this study were designed by *in silico* analysis. The extracellular domain (ECD) amino acid sequences of human HER2 (Accession No. P04626) and rat HER2 (Accession No. P06494) were retrieved from the UniProt protein database (https://www.uniprot.org/) in FASTA format [35]. Similarity of human and rat ECD HER2 proteins was analyzed by ExPASy, SIB Bioinformatics Resource Portal (https://embnet.vital-it.ch/software/LALIGN_form.html). Immune epitope database and analysis resource (IEDB) (https://www.iedb.org) was used for MHC I and MHC II epitope prediction. For the prediction of MHC I epitopes, 27 different alleles were used, covering 97% of HLA-A and HLA-B allelic variants in most ethnicities [36]. To predict MHC II epitopes, we selected a reference panel of 7 alleles, as described previously [37]. The IEDB server is based on the affinity for their receptor that can be inferred from the IC50 value and percentile rank assigned to each epitope. Peptides with the IC50 value < 50 nM, IC50 value < 500 nM, and IC50 value < 5000 nM were considered to have higher affinity, intermediate affinity, and low affinity, respectively, while peptides with the high percentile rank were considered to have low affinity [38]. The B cell epitopes were predicted using the SVMTriP (http://sysbio.unl.edu/SVMTriP/). The antigenicity of the obtained predictions was also determined by the Vaxijen v2.0 tool (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), and the predictions were considered both for T and B cell epitopes when predicted to have antigenic affinity by the BCEPRED tool (https://webs.iiitd.edu.in/raghava/bcepred/index.html) [39]. In addition, the ToxinPred tool (http://crdd.osdd.net/raghava/toxinpred/) was used to predict the toxicity of the epitopes [40]. Finally, predicted T cell and B cell rat epitopes were compared with predicted human epitopes, and sequences showing 80% or more similarity were selected as vaccine candidate epitopes.

2.2. DCs targeting

The ScFv sequence of the antibody targeting the DC-restricted antigen-uptake receptor DEC205 was selected for targeting DC. The ScFvDEC gene encodes variable region of the heavy chain (VH) and variable region of the light chain (VL). VL and VH sequences were previously published [41] and linked using the $(G4S)_3$ linker [42].

2.3. Designing the MeHer2 and ScFvDEC-MeHer2 proteins

The xenogeneic MeHer2 protein was generated by the seven epitopes linked together using the GGSG linker [43]. To enhance vaccine efficacy, the ScFvDEC sequence was introduced at the N terminus of the MeHer2 and was called ScFvDEC-MeHer2. The physicochemical features of ScFvDEC-MeHer2, ScFvDEC and MeHer2 proteins were computed by Expasy's ProtParam server (https://www.expasy.org/resources/protparam) [44]. The solubility of the proteins was predicted by the SOLpro server (http://scratch.proteomics.ics.uci.edu) [45], and the antigenicity of the proteins was also predicted by the ANTIGENpro (http://scratch.proteomics.ics.uci.edu) [46] and VaxiJen v2.0 (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) [47] servers. The proteins were analyzed for allergenicity using AllergenFP (http://ddg-pharmfac.net/AllergenFP/). The 3Dpro tool (http://scratch.proteomics.ics.uci.edu/) was used to predict the 3D structure of the ScFvDEC-MeHer2 protein [48]. The structure was viewed by the PyMOL molecular graphics system [49].

2.4. Construction of DNA vaccines

Codon optimization according to the preference of homo sapiens was performed for expression of the ScFvDEC-MeHer2 sequence (Gene Optimizer™, GeneArt, Regensburg, Germany) [50]. The codon-optimized sequence encoding ScFvDEC-MeHer2 was synthesized commercially and cloned into the pMX plasmid (Geneart AG, Regensburg, Germany). We amplified the ScFvDEC-MeHer2 sequence (1242 bp) with the primers sense 5′ CTCGAGGCCACCATGCAGGCCGT 3′ and anti-sense 5′ GAATTCTCAATGGTGATGGTGATGATGACGACCTGCTCTCTCGTCCAGGTCCAC 3′, the ScFvDEC sequence (780 bp) with the primers sense 5′ CTCGAGGCCACCATGCAGGCCGT 3′ and anti-sense 5′ GAATTCTCAATGGTGATGGTGATGATGACGACCTGCAGAAGAAACTGTCAGGG 3′, and the MeHer2 sequence (492 bp) with the primers sense 5′ CTCGAGGTGCCACTGCAGAGACTGAGAAT 3′ and anti-sense 5′ GAATTCTCAATGGTGATGGTGATGATGACGACCTGCTCTCTCGTCCAGGTCCAC 3′, respectively, with the Taq DNA polymerase (Thermoscientific, USA) according to the manufacturer's instructions. Primers were designed with a *Xho* I restriction site at the 5′ end of the coding region and an *EcoR* I site downstream of the termination codon. The *Xho* I and *EcoR* I sequences are underlined. At the 5′-end of the gene, the Kozak sequence and a start codon were added, while at the 3′-end a 6 × His-tag and a termination codon were added. PCR products were cloned into the pcDNA3.3-TOPO expression vector (Thermoscientific, USA) according to the manufacturer's instructions. The resulting plasmids were named pScFvDEC-MeHer2, pScFvDEC and pMeHer2. pEmpty plasmid without an insert was used as a negative control. Plasmids were transformed and grown in chemically competent TOP10 *E. coli* (Invitrogen, USA) and the positive colonies were confirmed by double digestion using *Xho*I (NEB, USA) and *Ecor*I (NEB, USA) and then with sequencing. After confirmation of the insert, plasmids were purified by the endotoxin-free plasmid DNA purification maxi kit (Qiagen, Germany) according to the manufacturer's protocol.

2.5. In vitro expression

Green-fluorescent protein (GFP) tag expression systems was used for protein expression confirmation of ScFvDEC-MeHer2 proteins in CHO K1 cells (ATCC® CCL-61™, USA). To include the GFP tag, the pScFvDEC-MeHer2 plasmid was double digested with *Xho* I and *EcoR* I, and then ScFvDEC-MeHer2 was cloned into the pIRES2-EGFP expression vector (BD Biosciences Clontech, USA) between restriction sites *Xho* I and *EcoR* I. The resulting plasmid was named pIRES2/EGFP-ScFvDEC-MeHer2. CHO K1 cells were transfected with 1 μg of pIRES2/EGFP-ScFvDEC-MeHer2 and precondensed with Lipofectamine-2000 (Thermoscientific, USA) in serum-free OPTI-MEM I + GlutaMAX I medium (Gibco, USA), as specified in the manufacturer's instructions. After 72 h of incubation at 37 °C, images of the GFP-ScFvDEC-MeHer2 expressing cells were acquired with a fluorescence microscope (Zeiss, Axio Vert.A1, Germany). The green fluorescence indicates the presence of ScFvDEC-MeHer2. The percentages of GFP-ScFvDEC-MeHer2 cells were analyzed and compared to PBS groups using flow cytometry (BD Accuri C5, USA).

HEK 293T cells (ATCC® CRL-3216™, USA) were used to determine the protein expression level of pScFvDEC-MeHer2, pScFvDEC, pMeHer2 and pEmpty plasmids. The cells were grown until 80% confluence in 6-well culture plates (Greiner Bio-One, Germany) and then transfected with pScFvDECME-Her2, pMEHer2 and pEmpty using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 9 μl of reagent was mixed with 3 μg of plasmid DNA in 750 μl of Opti-MEM (Gibco, USA), and the transfection complexes were incubated for 15 min at room temperature (RT). Then, the complexes were added to the cells, and forty-eight hours later, cell lysates were harvested using RIPA cell lysis buffer (Thermo scientific, USA).

For Western blot analysis, cell lysates were separated on a 12% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) and then transferred to a PVDF membrane (Immobilon-P, Millipore, USA). Blots were incubated with vaccinated mice sera pools (each pool contains serum samples of 5 mice from each group) as the primary antibody diluted to 1:100 for 1.5 h at RT, and then blots were probed with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma Aldrich, USA) diluted to 1:3000 for 1 h at RT. Next, the blots were visualized with alkaline phosphatase-developing buffer (0.1 M Na_2CO_3, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$) mixed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Fisher Scientific, USA) [51].

2.6. Mice and immunization schedule

All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Permit number: 2016-022). 6–8 weeks-old female BALB/c mice were used during the study. Groups of five mice were anesthetized and immunized intramuscularly (i.m) with pScFvDEC-MeHer2, pScFvDEC, pMeHer2, pEmpty (100 μg/dose), and PBS (100 μl), twice at a 3-week interval from the anterior tibial muscle. Blood samples were collected from the tail veins of the mice before the first immunization and three weeks after second immunization. Sera were separated by centrifugation at 3000 rpm for 10 min and stored at −20 °C until used.

2.7. Humoral immune response

The presence of anti-human HER2 IgG antibodies in vaccinated mice sera was initially analyzed by Western Blot using His tagged-Recombinant Human HER2 protein (SinoBiological, China) as described in section 2.5 [51]. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The PVDF membrane was incubated with 1:100 diluted vaccinated mouse sera pools (each pool contains serum samples of 5 mice of each group) as the primary antibody for 1.5 h at RT, and then blots were probed with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma Aldrich, USA) diluted to 1:3000 for 1 h at RT. Next, the blots were visualized with alkaline phosphatase-developing buffer mixed with BCIP and NBT. As a positive control, anti-His primary antibody (Sigma Aldrich, USA) was used.

The humoral immune responses in the pScFvDEC-MeHer2 vaccinated and control groups (pScFvDEC, pMeHer2, pEmpty, and PBS) were further determined by synthetic peptide ELISA as described with some modifications [52]. Briefly, for the detection of anti-MeHer2 specific total IgG, IgG1 and IgG2a antibodies, 96 well plates (Nunc, Thermo scientific, USA) were coated with 2 μg/well of the peptide pools (7 peptides comprising the MeHer2) diluted in deionized water overnight at 37 °C. Plates were then blocked with 0.5% BSA in PBS for 1 h at RT and washed thrice with PBS-T (0.1% Tween 20 in PBS). Then the plates were probed with mice sera (diluted to 1:33 with 0.5% BSA in PBS-T) and incubated for 2 h at RT. After washing, they were incubated with HRP conjugated IgG (1:3000 dilution), IgG1 (1:2500 dilution), or IgG2a (1:1500 dilution) antibodies (Sigma, USA) as secondary antibodies for 40 min at RT. The antigen-antibody complex was detected by the addition of TMB substrate (Thermo scientific, USA). The enzymatic reaction was stopped by 1 M H_2SO_4 and the optical density (OD) was measured at 450 nm using an automated microplate reader (Bio-Tek ELx808, USA).

2.8. Splenocyte isolation and stimulation

To investigate cellular immune responses in vaccinated and control groups, splenocytes were aseptically removed from mice 42 days after the first vaccination. Single cell suspensions were prepared as described [53]. Aliquots of 5×10^5 viable splenocytes were added to 96 well round bottom plates with 200 µl growth medium [RPMI 1640 (Gibco, USA) supplemented with 10% FCS (Hyclone, USA), 2 mM L-glutamine (Biochrom, Germany), 1% penicillin/streptomycin (Biosera, France), 0.1 mM non-essential amino acid (Gibco, USA) and 1 mM sodium pyruvate (Gibco, USA)] and stimulated with MeHer2 peptide pools for 72 h at 37 °C and 5% CO_2. The cells were incubated with 10 µg/ml of Concanavalin A (Sigma Aldrich, USA) as a positive control and with medium alone as a negative control.

2.9. Extracellular cytokine analysis

Splenocyte cell culture supernatants collected at 72 h were analyzed by ELISA for the presence of IL-4 and IFN-γ (Invitrogen, USA) according to the manufacturer's instructions. The detection limits for IL-4 and IFN-γ were 4 pg/ml and 15 pg/ml, respectively.

2.10. Flow cytometry

To evaluate the percentage of IL-4 secreting CD4, IFN-γ secreting CD4 and CD8 T cells induced by pScFvDEC-MeHer2, pScFvDEC and pMeHer2 vaccines, splenocytes from immunized mice were cultured as described above. Splenocytes were stained with Alexa flour 647 conjugated anti-CD3, FITC-conjugated anti-CD8, PerCP-cyanine 5.5 conjugated anti-CD4, PE-Cyanine 7 conjugated anti-IL-4 and PE-conjugated anti-IFN-γ antibodies (eBioscience, USA). A Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD Biosciences, USA) was used for the fixation and permeabilization of splenocytes. The cells were analyzed with a flow cytometer (LSRfortessa, BD, USA).

2.11. Statistical analyses

All data represents mean values with a standard deviation. Statistical analyses were performed using GraphPad Prism 9 software (GraphPad Software, USA). Statistically significant differences in the groups were assessed by one-tailed and two-tailed unpaired ANOVA. $P < 0.05$ was considered statistically significant.

Table 1
Predicted epitopes for the design of MeHer2.

Rat Epitope Sequences	Similarity Percentage to Human Sequence	Start-Stop	Type of Epitopes	Alleles	IC50	Rank or Score	Additional Properties
VPLQRLRIVRGTQL	100	94–107	MHC-I	B*07:02	637	1.3	VPLQRLRIV = B cell epitopeNon-toxin (−1.65)
				B*08:01	951	1.5	
			MHC-II	DRB4*01:01	30	0.1	
				DRB1*07:01	16	0.48	
				DRB1*15:01	192	2.8	
				DRB5*01:01	329	14	
FEDKYALAVLDN	91.67	108–119	B	–	–	1	Probable antigen (0.5868)Non-toxin (−1.22)
FGASCVTTCPYNYLSTEVGSCTL	91.3	FGASCVTTCPYNYL 292–305	MHC-I	A*01:01	226	0.31	Probable antigen (0.5537)Non-toxin (−0.80)
				A*68:01	314	1.3	
				A*30:01	1781	3	
				B*35:01	2490	2.3	
			MHC-II	DRB1*07:01	2073	42	
				DRB1*15:01	3769	62	
				DRB4*01:01	4527	68	
		PYNYLSTEVGSCTL 301–314	MHC-I	A*02:01	1513	5.4	Non-toxin (−1.22)
				A*23:01	3505	3.4	
				A*24:02	4958	4.1	
			MHC-II	DRB1*07:01	1125	29	
				DRB1*15:01	2995	56	
				DRB1*07:01	3086	44	
DGCKKIFGSLAFLPESFDGDPSSGIAPLRPEQ	84.38	366–397	B	–	–	1	Non-toxin (−0.92)
VPWDQLFRNPHQAL	100	481–494	MHC-I	B*07:02	44	0.17	Probable antigen (0.7107)Non-toxin (−0.51)
				B*35:01	784	0.44	
			MHC-II	DRB1*15:01	536	15	
				DRB3*02:02	678	8.2	
				DRB4*01:01	942	25	
				DRB1*07:01	1805	49	
				DRB3*01:01	2332	22	
				DRB1*03:01	2439	29	
				DRB5*01:01	2968	58	
SYMPIWKYPDEEGI	85.7	611–624	MHC-I	A*24:02	333	0.54	Probable antigen (0.7387)Non-toxin (−0.65)
				A*02:01	1451	5.3	
				A*23:01	1520	2	
				B*53:01	2185	1	
				B*51:01	4069	0.67	
			MHC-II	DRB5*01:01	3466	48	
				DRB1*15:01	3544	60	
QPCPINCTHSCVDLDER	88.24	626–642	MHC-I	B*07:02	2876	3.5	QPCPINCTHSCVDLDE = B cell epitopeProbable antigen (0.5188)Non-toxin (−0.95)
			MHC-II	DRB1*07:01	3521	42	

3. Results

3.1. Selection of epitopes and design of DC-targeted xenogeneic multi-epitope DNA vaccine

To induce cellular and humoral immune response synchronously, total seven peptides that contain 3T-cell epitopes, 2 linear B-cell epitopes, and 2T and B cell epitopes were selected for xenogeneic multi-epitope DNA vaccine development (Table 1). T cell epitopes have IC50 < 5000 and are probable antigens by the VaxiJen tool. B cell epitopes scored 1 by the SVMTriP tool or had antigenicity propensity by the BCEPRED tool were selected as immunogenic epitopes. All epitopes selected for the MeHer2 design did not have toxicity.

The peptides were combined using GGSG linkers. ScFvDEC specific for the DC antigen-uptake receptor DEC205 was introduced at the N terminus of the multi-epitope construct to increase the efficiency of MHC class I and MHC class II antigen presentation [31]. VL and VH sequences of ScFvDEC were combined by using the $(G4S)_3$ linker (Fig. 1A). The 3D structure of the ScFvDEC-MeHer2 protein obtained using the phyre2 web portal is given in Fig. 1B.

The physicochemical features of the ScFvDEC-MeHer2, ScFvDEC and MeHer2 proteins were determined by Expasy's ProtParam server (Table 2). The estimated half-life of the ScFvDEC-MeHer2 and ScFvDEC proteins was approximately 30 h in mammalian reticulocytes (*in vitro*), while the estimated half-life of MeHER2 was 100 h. ScFvDEC-MeHer2 and MeHER2 have solubility probabilities of 0.612008 and 0.546341, respectively, and ScFvDEC alone is not a soluble protein with a probability of 0.508218. Additionally, the antigenicity of ScFvDEC-MeHer2, ScFvDEC and MeHer2 proteins were computed as 0.929351, 0.968344 and 0.600583 by the ANTIGENpro server and as 0.6461, 0.7096 and 0.5267 by the Vaxijen. v2.0 server, respectively (Table 2).

After *in silico* analysis, codon optimization, cloning, digestion, and sequencing, the resulting plasmids were named pScFvDEC-MeHer2 as a vaccine vector, pScFvDEC and pMeHer2 as a control vector. pEmpty plasmid without insert was used as a negative control.

3.2. In vitro expression of the xenogeneic ScFvDEC-MeHer2 protein

The expression of ScFvDEC-MeHer2 protein in frame with GFP was analyzed by *in vitro* transfection of CHO K1 cells by pIRES-EGFP-ScFvDEC-MeHer2 plasmid. The green fluorescence indicates the presence of ScFvDEC-MeHer2 protein. As shown in Fig. 2A, GFP was observed in CHO K1 cells transfected with pIRES-EGFP-ScFvDEC-MeHer2 whereas any GFP was not observed in the PBS transfected cells (untreated group) using fluorescence microscopy. The percentages of GFP positive cells were analyzed in comparison to the PBS group using flow cytometry. The percentage of GFP-expressing cells was 35.95% ± 2.4.

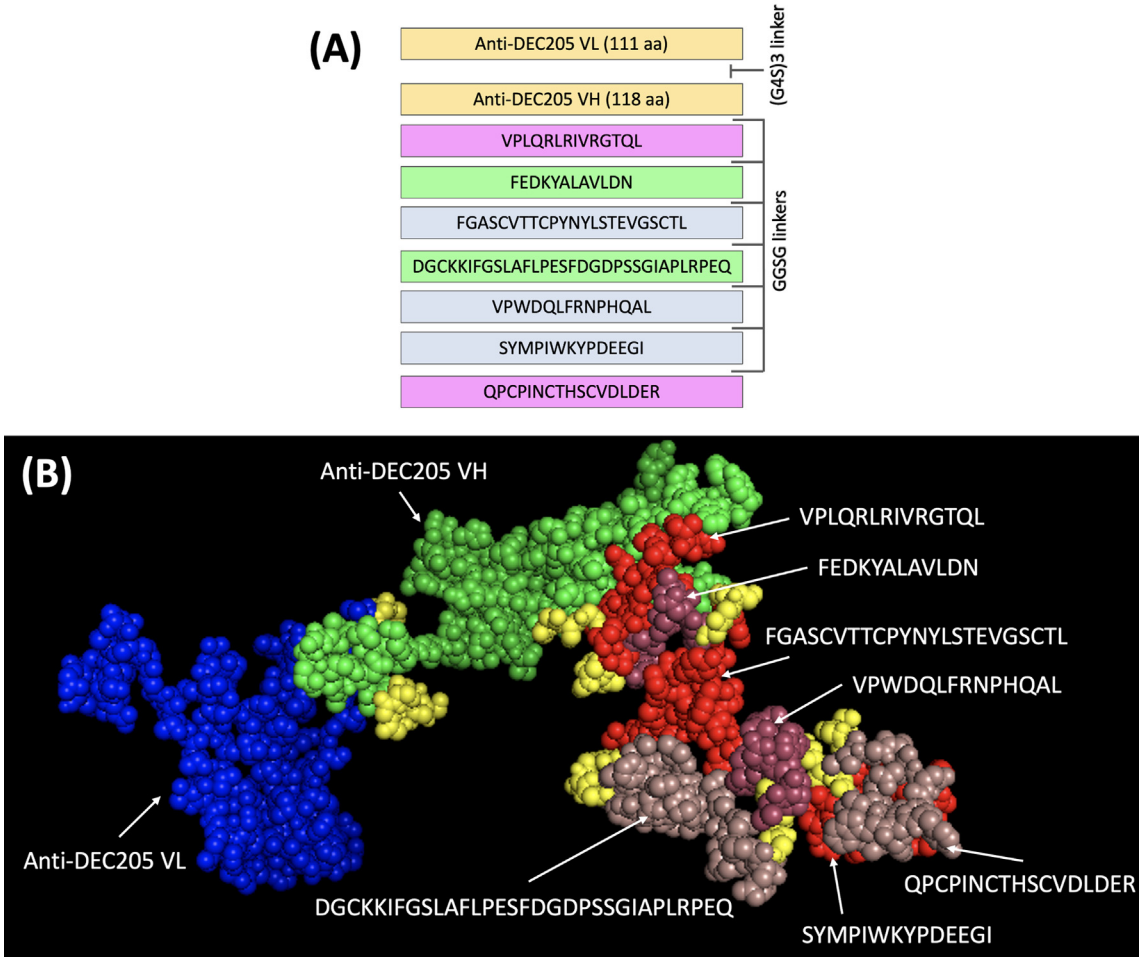

Fig 1. Epitope based vaccine design. (**A**) Schematic view of ScFvDEC-MeHer2 construct; ScFvDEC sequence [composed of anti-DEC205 light chain (Anti-DEC205 VL) and anti-DEC205 heavy chain (Anti-DEC205 VH)], T-cell epitopes (highlighted in grey color), B-cell epitopes (highlighted in green color) and both T and B cell epitopes (highlighted in pink color) (**B**) 3D view of ScFvDEC-MeHer2 construct by the PyMOL: the blue region represents the anti-DEC205 light chain, the green region the anti-DEC205 heavy chain, and the red region the xenogeneic MeHer2 region. Linkers are shown with yellow color.

Table 2
Physicochemical, solubility, antigenicity and allergenicity parameter results.

Proteins	Molecular Weight (kDa)	Theoretical PI	Total Number of Negatively Charged Residues (Asp + Glu)	Total Number of Positively Charged Residues (Arg + Lys)	The estimated half-life (hour)	Aliphatic Index	GRAVY	Solubility	Antigenicity by the Vaxijen	Antigenicity by the ANTIGENpro	Allergenicity
ScFvDEC-MeHer2	42.8	6.25	32	28	30	63.49	−0.305	0.612008/soluble	0.6461 (probable antigen)	0.929351	Probable non-allergen
ScFvDEC	26.78	8.29	16	18	30	62.45	−0.319	0.508218/ insoluble	0.7096 (probable antigen)	0.968344	Probable allergen
MeHer2	16.77	5.85	16	11	100	63.77	−0.4	0.546341/soluble	0.5267 (probable antigen)	0.600583	Probable non-allergen

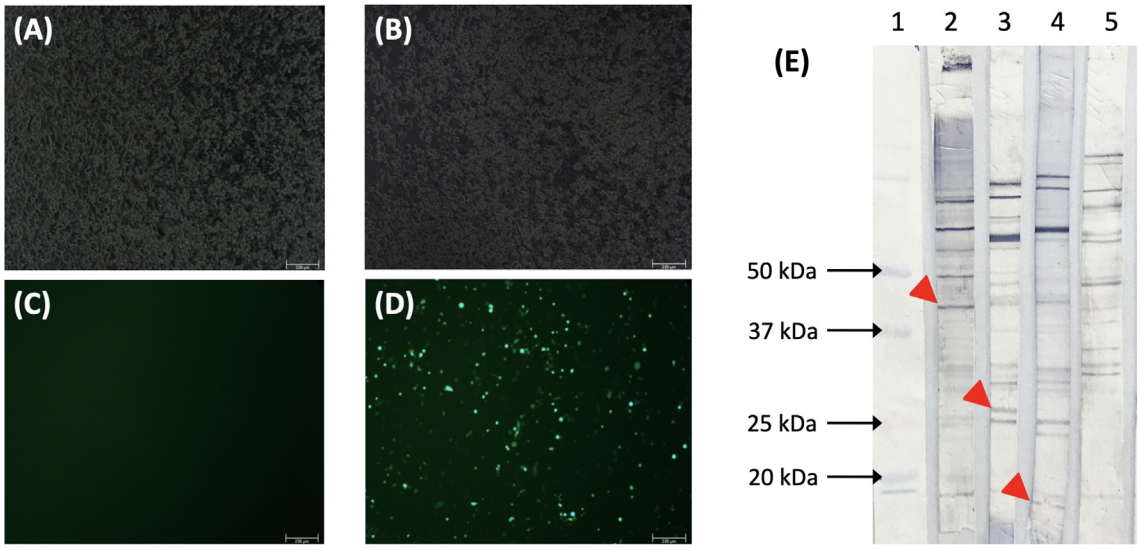

Fig 2. Expression of recombinant ScFvDEC-MeHer2 and MeHer2 proteins in CHO-K1 and 293T cells. **(A-D)** Fluorescence and light microscopy images of CHOK1 cells transfected with PBS (A, C) and pIRES2/EGFP-ScFvDEC-MeHer2 (B, D) 72 h after treatment (scale bar: 200 μm), **(E)** Western blot image of lysates obtained from HEK293 T cells transfected with pScFvDEC-MeHer2 (lane 2, 42.8 kDa), pScFvDEC (lane 3, 26.7 kDa), pMeHer2 (lane 4, 16.77 kDa) and pEmpty (lane 5). Red arrows represent recombinant proteins. Protein ladder is from Fermentas (lane 1). The original Western blot image can be reached from supplemental file.

Western blotting analysis showed that ScFvDEC-MeHer2, ScFvDEC and MeHer2 proteins were expressed in HEK 293T cells. The sera from immunized mice were complexed with 42.8 kDa, 26.7 kDa and 16.77 kDa proteins within the lysates of HEK 293T cells transfected with pScFvDEC-MeHer2, pScFvDEC and pMeHer2, respectively (Fig. 2B).

3.3. Humoral immune response induced by DNA immunization

To determine the humoral immune response induced by the pScFvDEC-MeHer2 vaccine and control groups (pScFvDEC, pMeHer2, pEmpty, and PBS), sera collected on days 0 and 42 were analyzed for the presence of antibodies against MeHer2 peptide pools using ELISA. As depicted in Fig. 3A, the levels of IgG generated in mice immunized with pScFvDEC-MeHer2 vaccine were significantly higher ($P < 0.0001$; ****) than the control groups after two immunizations (day 42). The pScFvDEC and pMeHer2 groups also showed a significant increase compared to the PBS ($P < 0.0001$; **** and $P = 0.0001$; ***) and pEmpty ($P < 0.0001$; **** and $P = 0.0003$; ***) groups. Any significant difference was not found at 42 days between mice immunized with pScFvDEC and pMeHer2 control.

According to Western Blot results, which were used to determine IgG antibodies against the recombinant Human HER2 protein, sera of mice immunized with pScFvDEC-MeHer2 and pMeHer2 revealed bands approximately equivalent to the human HER2 protein molecular weight ~ 110 kDa (the extracellular domain (Met 1-Thr 652) of human ErbB2 (NP_004439.2)). This band was not observed in the pScFvDEC, pEmpty, and PBS groups (Fig. 3C).

To determine whether a Th1 and/or Th2 response is induced in the immunized mice, MeHer2-specific IgG2a and IgG1 titers were analyzed by peptide ELISA. As shown in Fig. 3B, the level of IgG2a response against IgG1 was greater than or equal in the pScFvDEC-MeHer2 and pMeHer2 immunized mice. A comparison of IgG2a levels of pScFvDEC-MeHer2 with control groups showed that the DNA vaccine developed in this study induced significantly higher IgG2a response ($P < 0.01$; **). On the other hand, comparison of IgG1 levels of pScFvDEC-MeHer2 with control groups also showed a significant increase ($P < 0.05$; *) and any significance was not detected with the pMeHer2 group. These profiles of the antibodies suggest that the pScFvDEC-MeHer2 and pMeHer2 DNA vaccine strategies used in this study elicited an antigen specific Th1 humoral immune response.

3.4. Cellular immune response induced by DNA immunization

The percentages of CD4/IL-4, CD4/IFN-γ and CD8/IFN-γ secreting T cells from each group were evaluated by flow cytometry.

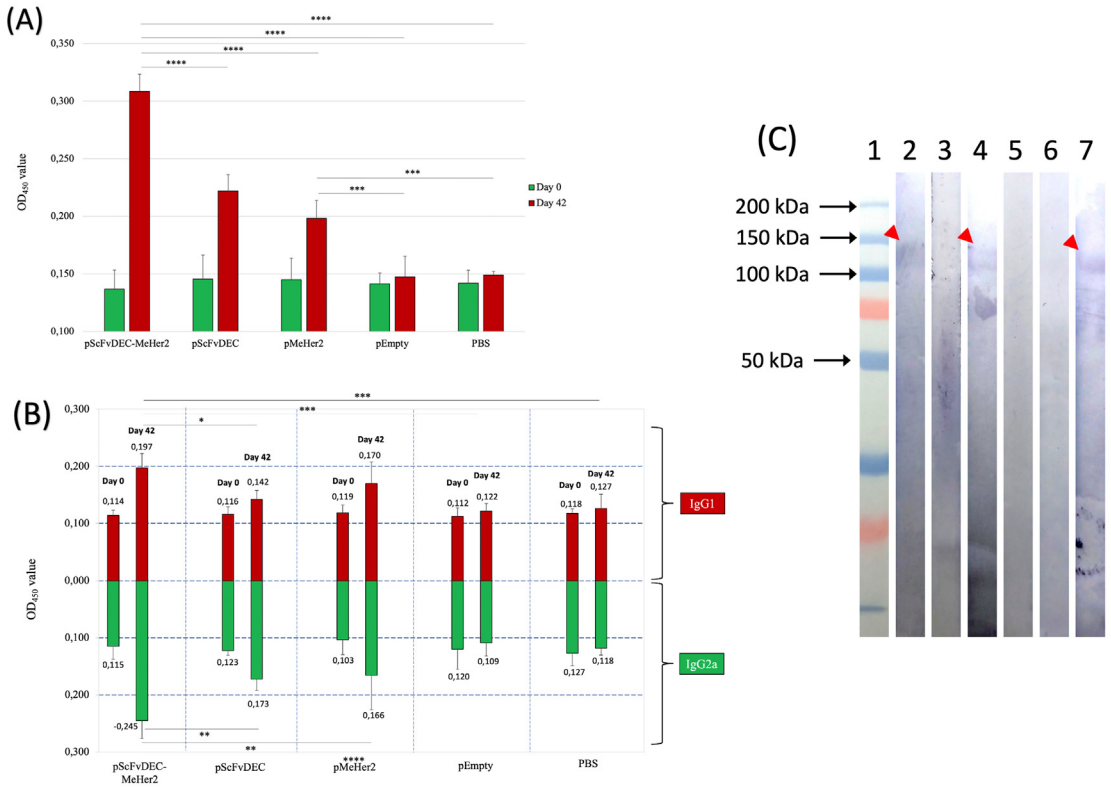

Fig 3. The humoral immune response according to the peptide ELISA and/or Western Blot **(A)** Specific OD450nm ± SD values of IgG obtained from mice sera **(B)** MeHer2 specific IgG1 and IgG2a levels. Each bar represents the mean ± SD. P-value indicators *** and **** refer to P < 0.001 and P < 0.0001 (C) Western blot showing the IgG response elicited by pScFvDEC-MeHer2 (lane 2), pScFvDEC (lane 3), pMeHer2 (lane 4), pEmpty (lane 5), and PBS (lane 6) against recombinant human HER2 proteins. Protein ladder is from Thermoscientific (lane 1) and lane 7 represents Anti-His antibody as a positive control. The original Western blot image can be reached from supplemental file.

As depicted in Fig. 4, the percentages of both CD4/IFN-γ and CD8/IFN-γ secreting T cells were higher in immunized mice with pScFvDEC-MeHer2 and pMeHer2 compared with the pScFvDEC, pEmpty and PBS control groups. In addition, the percentages of both CD4/IFN-γ and CD8/IFN-γ secreting T cells were higher in mice immunized with pScFvDEC-MeHer2 compared to the mice immunized with pMeHer2. The percentage of CD4/IL-4 secreting T cells among each group was not significant (P > 0.05).

To determine of cell-mediated immune responses, IFN-γ and IL-4 secreted from splenocytes stimulated with the MeHer2 peptide

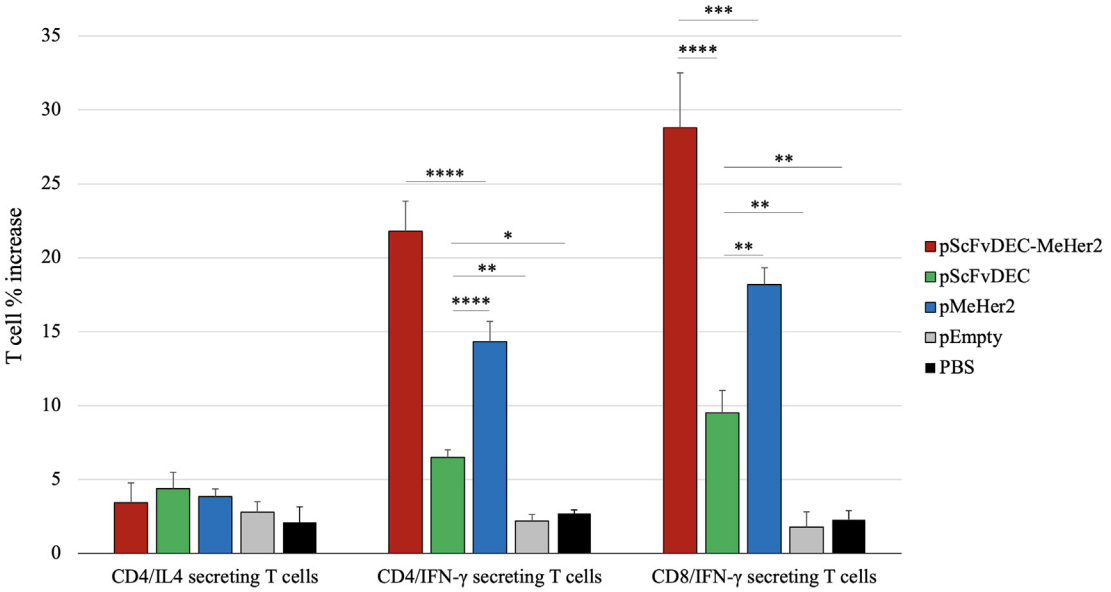

Fig 4. Percentages of CD4/IL4, CD4/IFN-γ and CD8/IFN-γ secreting T cells in splenocytes from immunized mice. P-value indicators *, **, *** and **** refer to P < 0.05, P < 0.01, P < 0.001 and P < 0.0001, respectively.

pools was analyzed using ELISA. The concentrations of IL-4 and IFN-γ produced by splenocytes are presented in Fig. 5. Culture supernatants of splenocyte stimulated with the MeHer2 peptide pools from mice immunized with pScFvDEC-MeHer2 and pMeHer2 produced significantly increased levels of IFN-γ compared to control groups (pScFvDEC, pEmpty and PBS) ($P < 0.0001$). In addition, the IFN-γ level of the pScFvDEC-MeHer2 vaccine group was 1.76 times higher than the pMeHer2 group ($P < 0.0001$). Extracellular IL-4 secretion was not detected in vaccinated groups or control groups. These results clearly support that vaccination with pScFvDECMeHer2 and pMeHer2 in mice enhanced significant IFN-γ production. Overall, cellular and humoral immune response data show that pScFvDECMeHer2 and pMeHer2 DNA vaccines induce Th1-biased immune responses.

4. Discussion

Vaccines as an active immunotherapy approach are used in the prevention and treatment of many diseases and have recently acquired prominence in cancer research. The most important reason for this is that controlling tumor formation using the patient's own immune system is more advantageous than radiotherapy and chemotherapy, which have significant negative effects on the patient's well-being [54]. In the present study, we generated a novel xenogeneic DNA vaccine (pScFvDEC-MeHer2) encoding a fusion protein comprised of the xenogeneic HER2 multi-epitope antigen and ScFvDEC. We demonstrated that targeting DCs using xenogeneic HER2 DNA vaccine technology significantly increases Th1 immunogenicity, which is very important in combating $HER2^+$ breast cancer.

The selective overexpression of HER2 on cancer tissues makes it an ideal target for the development of vaccines against $HER2^+$ breast cancer [55]. However, HER2 is tolerogenic, and because of this property, HER2 protein has low T cell avidity [56]. Different studies have proven that xenogeneic antigens, which are common to members of one species (rats) but not to members of other species (humans), have higher efficacy compared to autologous antigens. It is now accepted that the low avidity of antigens on cancer cells can be overcome using the xenogeneic DNA vaccine

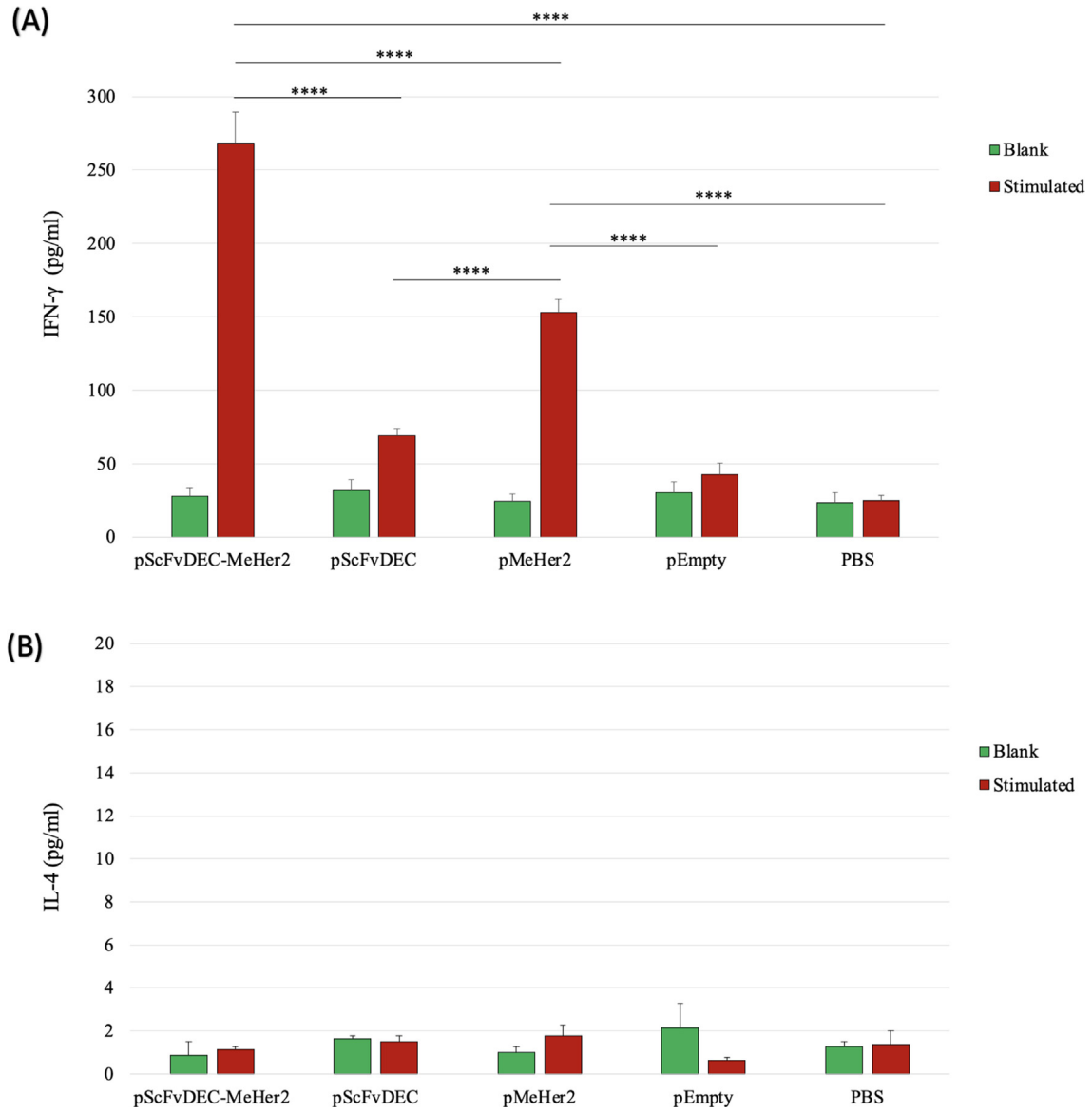

Fig 5. IFN-γ and IL-4 levels detected by ELISA from cell culture supernatants **(A)** the IFN-γ levels **(B)** the IL-4 levels. Right columns represent splenocytes stimulated with MeHer2 peptide pool.

strategy. In previous studies, DNA vaccines encoding xenogeneic HER2 proteins were developed to address this problem, and these DNA vaccines overcame the low T cell avidity [23,25,27]. In addition, the above-mentioned xenogeneic DNA vaccines were conventional DNA vaccines, and their immune protection was imperfect [26]. Therefore, a xenogeneic multi-epitope DNA vaccine strategy was used in the present study to provide increased safety and accurate immune focusing. The xenogeneic HER2 multi-epitope used as an antigen in the development of the DNA vaccine was designed by *in silico* approaches. Based on *in silico* analyses, seven peptides that contain three T-cell epitopes ($HER2_{292-314}$: FGASCVTTCPYNYLSTEVGSCTL; $HER2_{481-494}$: VPWDQLFRNPHQAL; $HER2_{611-624}$: SYMPIWKYPDEEGI), two linear B-cell epitopes ($HER2_{108-119}$: FEDKYALAVLDN; $HER2_{366-397}$: DGCKKIFGSLAFLPESF DGDPSSGIAPLRPEQ), and two T and B cell epitopes ($HER2_{94-107}$: VPLQRLRIVRGTQL; $HER2_{626-642}$: QPCPINCTHSCVDLDER) were selected for pMeHer2 vaccine development. $HER2_{366-397}$: DGCKK IFGSLAFLPESFDGDPSSGIAPLRPEQ epitope contains the extensively studied E75 epitope (HER2/neu 369–377: KIFGSLAFL) in clinical trials [57–59]. Any research has not been conducted with other epitopes used in design of our multi-epitope protein. The MeHer2 protein was generated by the seven epitopes combined by using GGSG linkers which are a flexible and short fusion linker [43]. This linker can minimize steric hindrance of each epitope and enhance epitope presentation to the host immune system [60]. As expected, pMeHer2 vaccine showed a significant humoral immune response as in previous HER2 DNA vaccine studies [61–64]. Furthermore, it showed a trend towards a Th1 response when the ratio of xenogeneic antigen-specific IgG2a/IgG1 levels and the percentages of T cells expressing both CD4/IFN-γ and CD8/IFN-γ were evaluated in mice immunized with pMeHer2. As a result, the xenogeneic multi-epitope DNA vaccine was found to be highly immunogenic and capable of eliciting both HER2 targeted humoral, and cell mediated immune responses.

CHOK1 cells and HEK293T cells were transfected by DNA vaccine and expressed the xenogeneic HER2 multi-epitope (Fig. 2). Our study did not address the question of whether uptake or expression of this construct by DEC205 + DCs or muscle cells *in vivo* after immunization. However, previous studies have shown that after intramuscular injection, the DNA vaccine was taken up and expressed by myocytes [65] and/or DCs [66] in mice. Myocytes lack MHC-II and co-stimulatory molecules and do not have access to T cells in lymphoid tissues, unlike DCs. Therefore, they are imperfect to present antigens and prime immune cells [67,68]. DCs are the most effective antigen-presenting cells that digest antigens into suitable peptides for presentation to the MHC-I and MHC-II pathways and play an important role in priming CD4 + and CD8 +T lymphocyte responses [69].

DNA vaccines have many advantages such as being safe, practical, and activating both the humoral and cellular immune responses, but they have poor immunogenicity in humans, which is a major drawback [23]. The most significant obstacle to the limited immunogenicity of DNA vaccines in humans is related to the difficulties of GMP scale up for clinical studies. Although approximately 5–20 mg of plasmid DNA is injected into an average-sized human, it may result with low expression of the antigen in antigen-presenting cells since the plasmid DNA can be degraded or most of the plasmid DNA cannot cross the nuclear barrier in cells [70]. Increasing antigen presentation by targeting dendritic cells can help overcome these issues resulting with poor immunogenicity of DNA vaccines. Several studies have shown that targeting antigen to DCs via DEC205 receptor by combining ScFvDEC in frame with vaccine antigen could enhance the efficacy of DNA vaccines and increase the presentation of antigen by both MHC class I and II molecules [31–34,71]. Therefore, we added ScFvDEC to the N terminus of the MeHer2 and constructed the pScFvDEC-MeHer2 DNA vaccine to investigate the immunogenicity in Balb/c mice. As anticipated, compared to pMeHer2 immunized mice, pScFvDEC-MeHer2 induced enhanced humoral and cellular immunity and displayed characteristic Th1 dominant response. The DNA vaccine development strategy defined herein for the first time which is the use of xenogeneic multi-epitope HER2 DNA vaccines that encode antigens targeted to DCs opens a new perspective to increase immunogenicity of DNA vaccines.

DNA vaccines have shown considerable promise in experimental animal models in terms of high protective immune responses against a variety of illnesses [72]. The weakness of this study is that the protective effect of the pScFvDEC-MeHer2 DNA vaccine was not investigated in $HER2^+$ breast cancer animal models. Future studies should evaluate the pScFvDEC-MeHer2 vaccine protection from breast cancer development in prophylactic or therapeutic vaccination settings in mice. In addition, the persistence of the immune response elicited by pScFvDEC-MeHer2 breast cancer vaccine or whether it induces memory type B/T cell responses can be further examined. Although our study was not designed to examine memory cell responses, it is worth noting that among the advantages of DNA vaccines is that they effectively induce both memory B and memory T-cell responses [72,73].

5. Conclusion

In the present study, a novel DNA vaccine (pScFvDEC-MeHer2) expressing xenogeneic multi-epitope HER2 in frame with ScFvDEC205 was developed. pScFvDEC-MeHer2 vaccine elicited significantly higher humoral and cellular immune response compared with to pMeHer2 vaccine. This dataset identifies pScFvDEC-MeHer2 as a potential DNA vaccine candidate, supporting further studies to be conducted on $HER2^+$ breast cancer animal models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.03.014.

References

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021;71 (3):209–49. https://doi.org/10.3322/caac.21660.

[2] Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. Clin Med Res 2009;7(1/2):4–13. https://doi.org/10.3121/cmr.2009.825.

[3] Dai X, Li T, Bai Z, Yang Y, Liu X, Zhan J, et al. Breast cancer intrinsic subtype classification, clinical use and future trends. Am J Cancer Res 2015;5 (10):2929–43.

[4] Nielsen DL, Andersson M, Kamby C. HER2-targeted therapy in breast cancer. Monoclonal antibodies and tyrosine kinase inhibitors. Cancer Treat Rev 2009;35(2):121–36. https://doi.org/10.1016/j.ctrv.2008.09.003.

[5] Ladjemi MZ, Jacot W, Chardès T, Pèlegrin A, Navarro-Teulon I. Anti-HER2 vaccines: new prospects for breast cancer therapy. Cancer Immunol

Immunother 2010;59(9):1295–312. https://doi.org/10.1007/s00262-010-0869-2.
[6] Modjtahedi H, Cho BC, Michel MC, Solca F. A comprehensive review of the preclinical efficacy profile of the ErbB family blocker afatinib in cancer. Naunyn Schmiedebergs Arch Pharmacol 2014;387(6):505–21. https://doi.org/10.1007/s00210-014-0967-3.
[7] Tang Y, Wang Y, Kiani MF, Wang B. Classification, Treatment Strategy, and Associated Drug Resistance in Breast Cancer. Clin Breast Cancer 2016;16(5):335–43. https://doi.org/10.1016/j.clbc.2016.05.012.
[8] Dias AS, Almeida CR, Helguero LA, Duarte IF. Metabolic crosstalk in the breast cancer microenvironment. Eur J Cancer 2019;121:154–71. https://doi.org/10.1016/j.ejca.2019.09.002.
[9] Pallerla S, Abdul AURM, Comeau J, Jois S. Cancer Vaccines, Treatment of the Future: With Emphasis on HER2-Positive Breast Cancer. Int J Mol Sci 2021;22(2):779. https://doi.org/10.3390/ijms22020779.
[10] Song Q, Zhang CD, Wu XH. Therapeutic cancer vaccines: From initial findings to prospects. Immunol Lett 2018;196:11–21. https://doi.org/10.1016/j.imlet.2018.01.011.
[11] Disis ML, Calenoff E, McLaughlin G, Murphy AE, Chen W, Groner B, et al. Existent T-cell and antibody immunity to HER-2/neu protein in patients with breast cancer. Cancer Res 1994;54(1):16–20.
[12] Kitano S, Kageyama S, Nagata Y, Miyahara Y, Hiasa A, Naota H, et al. HER2-specific T-cell immune responses in patients vaccinated with truncated HER2 protein complexed with nanogels of cholesteryl pullulan. Clin Cancer Res 2006;12(24):7397–405. https://doi.org/10.1158/1078-0432.CCR-06-1546.
[13] Curigliano G, Romieu G, Campone M, Dorval T, Duck L, Canon JL, et al. A phase I/II trial of the safety and clinical activity of a HER2-protein based immunotherapeutic for treating women with HER2-positive metastatic breast cancer. Breast Cancer Res Treat 2016;156(2):301–10. https://doi.org/10.1007/s10549-016-3750-y.
[14] Gillogly ME, Kallinteris NL, Xu M, Gulfo JV, Humphreys RE, Murray JL. Ii-Key/HER-2/neu MHC class-II antigenic epitope vaccine peptide for breast cancer. Cancer Immunol Immunother 2004;53(6):490–6. https://doi.org/10.1007/s00262-003-0463-y.
[15] Mittendorf EA, Storrer CE, Foley RJ, Harris K, Jama Y, Shriver CD, et al. Evaluation of the HER2/ neu -derived peptide GP2 for use in a peptide-based breast cancer vaccine trial: Peptide-Based GP2 Vaccine Strategy. Cancer 2006;106(11):2309–17.
[16] Clifton GT, Gall V, Peoples GE, Mittendorf EA. Clinical Development of the E75 Vaccine in Breast Cancer. Breast Care (Basel) 2016;11(2):116–21. https://doi.org/10.1159/000446097.
[17] Brown TA, Mittendorf EA, Hale DF, Myers JW, Peace KM, Jackson DO, et al. Prospective, randomized, single-blinded, multi-center phase II trial of two HER2 peptide vaccines, GP2 and AE37, in breast cancer patients to prevent recurrence. Breast Cancer Res Treat 2020;181(2):391–401.
[18] Czerniecki BJ, Koski GK, Koldovsky U, Xu S, Cohen PA, Mick R, et al. Targeting HER-2/neu in early breast cancer development using dendritic cells with staged interleukin-12 burst secretion. Cancer Res 2007;67(4):1842–52. https://doi.org/10.1158/0008-5472.CAN-06-4038.
[19] Koski GK, Koldovsky U, Xu S, Mick R, Sharma A, Fitzpatrick E, et al. A novel dendritic cell-based immunization approach for the induction of durable Th1-polarized anti-HER-2/neu responses in women with early breast cancer. J Immunother 2012;35(1):54–65. https://doi.org/10.1097/CJI.0b013e318235f512.
[20] Sharma A, Koldovsky U, Xu S, Mick R, Roses R, Fitzpatrick E, et al. HER-2 pulsed dendritic cell vaccine can eliminate HER-2 expression and impact ductal carcinoma in situ. Cancer 2012;118(17):4354–62. https://doi.org/10.1002/cncr.26734.
[21] Norell H, Poschke I, Charo J, Wei WZ, Erskine C, Piechocki MP, et al. Vaccination with a plasmid DNA encoding HER-2/neu together with low doses of GM-CSF and IL-2 in patients with metastatic breast carcinoma: a pilot clinical trial. J Transl Med 2010;8:53. https://doi.org/10.1186/1479-5876-8-53.
[22] Diaz CM, Chiappori A, Aurisicchio L, Bagchi A, Clark J, Dubey S, et al. Phase 1 studies of the safety and immunogenicity of electroporated HER2/CEA DNA vaccine followed by adenoviral boost immunization in patients with solid tumors. J Transl Med 2013;11:62. https://doi.org/10.1186/1479-5876-11-62.
[23] Quaglino E, Riccardo F, Macagno M, Bandini S, Cojoca R, Ercole E, et al. Chimeric DNA Vaccines against ErbB2+ Carcinomas: From Mice to Humans. Cancers (Basel) 2011;3(3):3225–41. https://doi.org/10.3390/cancers3033225.
[24] Occhipinti S, Sponton L, Rolla S, Caorsi C, Novarino A, Donadio M, et al. Chimeric rat/human HER2 efficiently circumvents HER2 tolerance in cancer patients. Clin Cancer Res 2014;20(11):2910–21. https://doi.org/10.1158/1078-0432.CCR-13-2663.
[25] Srinivasan R, Wolchok JD. Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines. J Transl Med 2004;2(1):12. https://doi.org/10.1186/1479-5876-2-12.
[26] Tian L, Wang HN, Lu D, Zhang YF, Wang T, Kang RM. The immunoreactivity of a chimeric multi-epitope DNA vaccine against IBV in chickens. Biochem Biophys Res Commun 2008;377(1):221–5. https://doi.org/10.1016/j.bbrc.2008.09.125.
[27] Lopes A, Vandermeulen G, Préat V. Cancer DNA vaccines: current preclinical and clinical developments and future perspectives. J Exp Clin Cancer Res 2019;38(1):146. https://doi.org/10.1186/s13046-019-1154-7.
[28] Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. Curr Opin Immunol 2003;15(4):461–70. https://doi.org/10.1016/S0952-7915(03)00083-9.
[29] Wang X, Xu W, Tong D, Ni J, Gao H, Wang Y, et al. A chimeric multi-epitope DNA vaccine elicited specific antibody response against severe acute respiratory syndrome-associated coronavirus which attenuated the virulence of SARS-CoV *in vitro*. Immunol Lett 2008;119(1–2):71–7. https://doi.org/10.1016/j.imlet.2008.04.005.
[30] Pumchan A, Krobthong S, Roytrakul S, Sawatdichaikul O, Kondo H, Hirono I, et al. Novel Chimeric Multiepitope Vaccine for Streptococcosis Disease in Nile Tilapia (Oreochromis niloticus Linn.). Sci Rep 2020;10(1). https://doi.org/10.1038/s41598-019-57283-0.
[31] Nchinda G, Kuroiwa J, Oks M, Trumpfheller C, Park CG, Huang Y, et al. The efficacy of DNA vaccination is enhanced in mice by targeting the encoded protein to dendritic cells. J Clin Invest 2008;118(4):1427–36. https://doi.org/10.1172/JCI34224.
[32] Grossmann C, Tenbusch M, Nchinda G, Temchura V, Nabi G, Stone GW, et al. Enhancement of the priming efficacy of DNA vaccines encoding dendritic cell-targeted antigens by synergistic toll-like receptor ligands. BMC Immunol 2009;10:43. https://doi.org/10.1186/1471-2172-10-43.
[33] Nchinda G, Amadu D, Trumpfheller C, Mizenina O, Uberla K, Steinman RM. Dendritic cell targeted HIV gag protein vaccine provides help to a DNA vaccine including mobilization of protective CD8+ T cells. Proc Natl Acad Sci USA 2010;107(9):4281–6. https://doi.org/10.1073/pnas.1000621107.
[34] Hua Y, Jiao YY, Ma Y, Peng XL, Fu YH, Zhang XJ, et al. Enhanced humoral and CD8+ T cell immunity in mice vaccinated by DNA vaccine against human respiratory syncytial virus through targeting the encoded F protein to dendritic cells. Int Immunopharmacol 2017;46:62–9. https://doi.org/10.1016/j.intimp.2017.02.023.
[35] Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, et al. UniProt: the Universal Protein knowledgebase. Nucleic Acids Res 2004;32:D115–9. https://doi.org/10.1093/nar/gkh131.
[36] Weiskopf D, Angelo MA, de Azeredo EL, Sidney J, Greenbaum JA, Fernando AN, et al. Comprehensive analysis of dengue virus-specific responses supports an HLA-linked protective role for CD8 + T cells. Proc Natl Acad Sci USA 2013;110(22).
[37] Paul S, Lindestam Arlehamn CS, Scriba TJ, Dillon MB, Oseroff C, Hinz D, et al. Development and validation of a broad scheme for prediction of HLA class II restricted T cell epitopes. J Immunol Methods 2015;422:28–34. https://10.1016/j.jim.2015.03.022.
[38] Khatoon N, Pandey RK, Prajapati VK. Exploring Leishmania secretory proteins to design B and T cell multi-epitope subunit vaccine using immunoinformatics approach. Sci Rep 2017;7(1):8285. , https://10.1038/s41598-017-08842-w.
[39] Saha S, Raghava GPS. BcePred:Prediction of Continuous B-Cell Epitopes in Antigenic Sequences Using Physico-chemical Properties. In: Nicosia G, Cutello V, Bentley PJ, Timis J, editors. Springer; 2004. p. 197–204.
[40] Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Raghava GP. In silico approach for predicting toxicity of peptides and proteins. PLoS One 2013;8(9):e73957. , https://10.1371/journal.pone.0073957.
[41] Demangel C, Zhou J, Choo AB, Shoebridge G, Halliday GM, Britton WJ. Single chain antibody fragments for the selective targeting of antigens to dendritic cells. Mol Immunol 2005;42(8):979–85. https://doi.org/10.1016/j.molimm.2004.09.034.
[42] Yuan J, Kashiwagi S, Reeves P, Nezivar J, Yang Y, Arrifin NH, et al. A novel mycobacterial Hsp70-containing fusion protein targeting mesothelin augments antitumor immunity and prolongs survival in murine models of ovarian cancer and mesothelioma. J Hematol Oncol 2014;7:15. https://doi.org/10.1186/1756-8722-7-15.
[43] Zeng Q. Polypeptide vaccine; 2013. WO 2013036201. https://patentimages.storage.googleapis.com/5b/c9/a1/9b1458d57f59f9/WO2013036201A1.pdf [accession date: 10.02.2022].
[44] Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 2003;31:3784–8. https://doi.org/10.1093/nar/gkg563.
[45] Magnan CN, Randall A, Baldi P. SOLpro: accurate sequence-based prediction of protein solubility. Bioinformatics 2009;25:2200–7. https://doi.org/10.1093/bioinformatics/btp386.
[46] Magnan CN, Zeller M, Kayala MA, Vigil A, Randall A, Felgner PL, et al. High-throughput prediction of protein antigenicity using protein microarray data. Bioinformatics 2010;26(23):2936–43. https://doi.org/10.1093/bioinformatics/btq551.
[47] Doytchinova IA, Flower DR. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinf 2007;8:4. https://doi.org/10.1186/1471-2105-8-4.
[48] Cheng J, Randall AZ, Sweredoski MJ, Baldi P. SCRATCH: a protein structure and structural feature prediction server. Nucleic Acids Res 2005;33:W72–6. https://doi.org/10.1093/nar/gki396.
[49] Pymol DWL. An open-source molecular graphics tool. CCP4 Newsletter Protein Crystallogr 2002;40:82–92.
[50] Muthumani K, Flingai S, Wise M, Tingey C, Ugen KE, Weiner DB. Optimized and enhanced DNA plasmid vector based in vivo construction of a neutralizing anti-HIV-1 envelope glycoprotein Fab. Hum Vaccin Immunother 2013;9(10):2253–62. https://doi.org/10.4161/hv.26498.
[51] Karakavuk M, Can H, Gül A, Döşkaya AD, Alak SE, Ün C, et al. GRA8 DNA vaccine formulations protect against chronic toxoplasmosis. Microb Pathog 2021;158:. , https://10.1016/j.micpath.2021.105016105016.
[52] Gülce-İz S, Elaldı N, Can H, Şahar EA, Karakavuk M, Gül A, et al. Development of a novel recombinant ELISA for the detection of Crimean-Congo hemorrhagic

fever virus IgG antibodies. Sci Rep 2021;11(1):5936. https://doi.org/10.1038/s41598-021-85323-1.
[53] Döşkaya M, Kalantari-Dehaghi M, Walsh CM, Hiszczyńska-Sawicka E, Davies DH, Felgner PL, et al. GRA1 protein vaccine confers better immune response compared to codon-optimized GRA1 DNA vaccine. Vaccine 2007;25(10):1824–37. https://doi.org/10.1016/j.vaccine.2006.10.060.
[54] Gül A, Erkunt-Alak S, Gül C, Karakavuk T, Can H, Karakavuk M, et al. Breast Cancer Vaccines: Current Status and Future Approach. In: Atta-ur-Rahman FRS, editor. Frontiers in Clinical Drug Research-Anti-Cancer Agents, Bentham Books imprint; 2021. p. 108–41.
[55] Marchini C, Kalogris C, Garulli C, Pietrella L, Gabrielli F, Curcio C, et al. Tailoring DNA Vaccines: Designing Strategies Against HER2-Positive Cancers. Front Oncol 2013;3:122. https://doi.org/10.3389/fonc.2013.00122.
[56] Kiessling R, Wei WZ, Herrmann F, Lindencrona JA, Choudhury A, Kono K, et al. Cellular immunity to the Her-2/neu protooncogene. Adv Cancer Res 2002;85:101–44. https://doi.org/10.1016/S0065-230X(02)85004-7.
[57] Murray JL, Gillogly ME, Przepiorka D, Brewer H, Ibrahim NK, Booser DJ, et al. Toxicity, immunogenicity, and induction of E75-specific tumor-lytic CTLs by HER-2 peptide E75 (369–377) combined with granulocyte macrophage colony-stimulating factor in HLA-A2+ patients with metastatic breast and ovarian cancer. Clin Cancer Res 2002;8(11):3407–18.
[58] Mittendorf EA, Clifton GT, Holmes JP, Clive KS, Patil R, Benavides LC, et al. Clinical trial results of the HER-2/neu (E75) vaccine to prevent breast cancer recurrence in high-risk patients: from US Military Cancer Institute Clinical Trials Group Study I-01 and I-02. Cancer 2012;118(10):2594–602. https://doi.org/10.1002/cncr.26574.
[59] Mittendorf EA, Clifton GT, Holmes JP, Schneble E, van Echo D, Ponniah S, et al. Final report of the phase I/II clinical trial of the E75 (nelipepimut-S) vaccine with booster inoculations to prevent disease recurrence in high-risk breast cancer patients. Ann Oncol 2014;25(9):1735–42. https://doi.org/10.1093/annonc/mdu211.
[60] Farhadi T, Nezafat N, Ghasemi Y, Karimi Z, Hemmati S, Erfani N. Designing of complex multi-epitope peptide vaccine based on omps of *Klebsiella pneumonia*: an *in-silico* approach. Int J Pept Res Ther 2015;21:325–41. https://doi.org/10.1007/s10989-015-9461-0.
[61] Amici A, Smorlesi A, Noce G, Santoni G, Cappelletti P, Capparuccia L, et al. DNA vaccination with full-length or truncated neu induces protective immunity against the development of spontaneous mammary tumors in HER-2/neu transgenic mice. Gene Ther 2000;7(8):703–6. https://doi.org/10.1038/sj.gt.3301151.
[62] Jacob J, Radkevich O, Forni G, Zielinski J, Shim D, Jones RF, et al. Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cell Immunol 2006;240(2):96–106. https://doi.org/10.1016/j.cellimm.2006.07.002.
[63] Jacob JB, Quaglino E, Radkevich-Brown O, Jones RF, Piechocki MP, Reyes JD, et al. Combining human and rat sequences in her-2 DNA vaccines blunts immune tolerance and drives antitumor immunity. Cancer Res 2010;70(1):119–28. https://doi.org/10.1158/0008-5472.CAN-09-2554.
[64] Quaglino E, Mastini C, Amici A, Marchini C, Iezzi M, Lanzardo S, et al. A better immune reaction to Erbb-2 tumors is elicited in mice by DNA vaccines encoding rat/human chimeric proteins. Cancer Res 2010;70(7):2604–12. https://doi.org/10.1158/0008-5472.CAN-09-2548.
[65] Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, et al. Direct gene transfer into mouse muscle in vivo. Science 1990;247:1465–8. https://doi.org/10.1126/science.
[66] Casares S, Inaba K, Brumeanu TD, Steinman RM, Bona CA. Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. J Exp Med 1997;186(9):1481–6. https://doi.org/10.1084/jem.186.9.1481.
[67] Lindquist RL, Shakhar G, Dudziak D, Wardemann H, Eisenreich T, Dustin ML, et al. Visualizing dendritic cell networks in vivo. Nat Immunol 2004;5(12):1243–50. https://doi.org/10.1038/ni1139.
[68] Wiendl H, Hohlfeld R, Kieseier BC. Muscle-derived positive and negative regulators of the immune response. Curr Opin Rheumatol 2005;17(6):714–9. https://doi.org/10.1097/01.bor.0000184164.69181.ca.
[69] Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nat Rev Immunol 2012;12(8):557–69. https://doi.org/10.1038/nri3254.
[70] Hobernik D, Bros M. DNA Vaccines-How Far From Clinical Use? Int J Mol Sci 2018;19(11):3605. https://doi.org/10.3390/ijms19113605.
[71] Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 2002;196(12):1627–38.
[72] Huang J, Ma R, Wu CY. Immunization with SARS-CoV S DNA vaccine generates memory CD4+ and CD8+ T cell immune responses. Vaccine 2006;24(23):4905–13. https://doi.org/10.1016/j.vaccine.2006.03.058.
[73] Mwangi W, Brown WC, Splitter GA, Davies CJ, Howard CJ, Hope JC, et al. DNA vaccine construct incorporating intercellular trafficking and intracellular targeting motifs effectively primes and induces memory B- and T-cell responses in outbred animals. Clin Vaccine Immunol 2007;14(3):304–11. https://doi.org/10.1128/CVI.00363-06.