Adjuvant activity of GP96 C-terminal domain towards Her2/*neu* DNA vaccine is fusion direction-dependent

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Abstract The Her2 is one of tumor-associated antigens (TAA), regarded as an ideal target of immunotherapy. DNA encoding full-length or truncated rat Her2/*neu* have shown protective and therapeutics potentials against Her2/*neu*-expressing mammary tumors. However, the efficacy of active vaccination is limited since Her2 is a self-tolerated antigen. Hence, new strategies are required to enhance both the quality and quantity of the immune response against Her2-expressing tumors. Many studies have used Her2/*neu* gene with cytokine or other molecules involved in regulation of immune response to enhance the potency of Her2/*neu* DNA vaccines. Some studies fused adjuvant gene to C-terminal domain of Her2/*neu* gene, while others fused the adjuvant gene N-terminally to Her2/*neu* gene, but no comparison on how direction of fusion could affect efficiency of DNA vaccine has ever been made. Based on previous reports demonstrating potent adjuvant activity of gp96 C-terminal domain, we chose it as adjuvant. The aim of this study was to investigate if direction of fusion could affect adjuvant activity of gp96 C-terminal domain or potency of Her2/*neu* DNA vaccination. To do so, we fused C-terminal domain of gp96 to downstream or C-terminal end of transmembrane and extracellular domain (TM+ECD) of rat Her2/*neu* and resultant immune response to DNA vaccination was evaluated. The results were compared with that of N-terminally fusion of gp96 C-terminal domain to TM+ECD of rat Her2/*neu*. Our results revealed that adjuvant activity of gp96 C-terminal domain is enhanced when fused N-terminally to TM+ECD of rat Her2/*neu*. It suggests that adjuvant activity of gp96 C-terminal domain towards Her2/*neu* is fusion direction-dependent.

Keywords Her2 · gp96 C-terminal domain
Regulatory T cells · Fusion direction-dependent
DNA vaccine

Introduction

The Her2/ErbB2/*neu* proto-oncogene is one of the TAAs, regarded as an ideal target of immunotherapy because of its elevated expression in tumors, cell surface localization, and involvement in cancer progression/worsening prognosis. It is a member of the epidermal growth factor receptor family and involved in a variety of malignancies, including breast, ovarian, and gastric carcinomas (Ishikawa et al. 1997; Slamon et al. 1989). DNA encoding full-length or truncated rat Her2/*neu* have shown to have protective and therapeutics potentials against Her2/*neu*-expressing mammary tumors (Lin et al. 2004; Quaglino et al. 2005; Rovero et al. 2000; Wei et al. 1999). However, plasmid-based DNA vaccines have not been potent when translated to human clinical use. Hence, new strategies are required to enhance both the quality and quantity of the immune response against Her2-expressing tumors. Many studies have used Her2 gene with cytokine or other molecules involved in regulation of immune response to enhance the potency of Her2/*neu* DNA vaccines (Kim et al. 2005; Lin et al. 2004; Orlandi et al. 2007). Some studies fused adjuvant gene to C-terminal of Her2/*neu* gene (Kim et al. 2005; Lin et al. 2004), while others fused the adjuvant gene to N-terminal end of Her2/*neu* gene (Orlandi et al. 2007; Pakravan et al. 2010a, b), but no comparison on how direction of fusion could affect efficiency of Her2/*neu* DNA vaccine has ever been made.

We previously (Pakravan et al. 2010a) demonstrated that fusion, but not co-administration, of gp96 C-terminal domain to TM+ECD of rat Her2/*neu* led to better inhibition of tumor

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growth. It prompted us to present results indicating increased efficiency of TM+ECD of rat Her2/neu DNA vaccine, when fused C-terminally to gp96 C-terminal domain. In this article, the result on the efficiency of C-terminally fusion of gp96 C-terminal to TM+ECD of rat Her2/neu DNA vaccine is presented. It is noteworthy to remind that this study was conducted at the same time of the previous report (Pakravan et al. 2010a). The results of N-terminally fusion of gp96 C-terminal to TM+ECD of rat Her2/neu were published previously (Pakravan et al. 2010a) and statistical analysis with C-terminally fusion of gp96 C-terminal to TM+ECD of rat Her2/neu have been mentioned here for comparison.

Material and methods

Mice

Female BALB/c mice at the age of 8–10 weeks were purchased from Pasteur Institute, Tehran, Iran. Given free access to food and water, mice were housed for 1 week before experiments and maintained in a good standard condition. All experiments were done according to Animal Care and Use Protocol of Tarbiat Modares University.

Cell line

TUBO is a cloned cell line (kindly provided by Professor Federica Cavallo, University of Turin, Turin, Italy) derived from a lobular carcinoma that arose in a female BALB/c mouse transgenic for the rat Her2/neu oncogene (BALB-NeuT mouse). TUBO cells are poorly immunogenic in normal BALB/c mice; they do not induce anti-rat Her2/neu antibodies or any detectable Cytotoxic T lymphocyte (CTL) response (Rovero et al. 2000). The cells were cultured in DMEM (CytoGen, Germany) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (BioSera Ltd, UK), and 20% heat-inactivated fetal bovine serum (Gibco BRL, U.K) and maintained at 37°C, 5% CO₂.

A suspension of 0.15 ml containing 3×10^5 of TUBO cells was injected subcutaneously.

The cages were coded, and neoplastic masses were measured using calipers. Vaccination was commenced 13 days after tumor implantation.

DNA expression vectors and vaccination

TM+ECD of rat Her2/neu (kindly provided by Professor Federica Cavallo; GenBank Accession no. NM_017003) was located between Hind III/Eco RI within pcDNA3* plasmid (pHer2). pcDNA3* was a derivation of the pcDNA3 plasmid (Invitrogen) by deleting the SV40 promoter, neomycin resistance gene, and SV40 poly(A) (Rovero et al. 2000).

Subsequently, half downstream (~1,200 bp) of human gp96 C-terminal domain (kindly provided by Professor Brian Seed, Harvard Medical School, Boston, Massachusetts, USA; GenBank Accession no. NM_003299) was cloned into pHer2, downstream of Her2/neu (pHer2/CT). Preparation of pCT/Her2 was described previously (Pakravan et al. 2010a). The expression of each construct was evaluated by cloning of green fluorescent gene at the end of gene, as described previously (Pakravan et al. 2010c). All of the constructs were confirmed by sequencing.

Each construct was amplified using DH5α strain of *Escherichia coli* in Luria–Bertani medium. Large-scale preparation of the plasmid was performed according to standard polyethylene glycol (PEG) precipitation method (Sambrook and Russell 2001).

Vaccination

Mice (five in each group) were grouped into six cages, and at day 0, the tumor was implanted. Starting 12 days after tumor challenge, the animals were intramuscularly vaccinated with 100 µg of DNA vaccine three times at weekly intervals. The treatment groups were saline, empty plasmids, pHer2, pCT/Her2, or pHer2/CT. Mice were killed 2 weeks after the last vaccination. The mean diameter of tumor was recorded every other day from the beginning of vaccination until the animals were killed.

Proliferation assay of spleen cells

An extract of TUBO cells was prepared by making a suspension of a tumor mass, passing through mesh, freeze/thawing, sonication, and finally filtering the extract. The concentration of the extract was then determined at 595 nm and stored frozen at −20°C until used.

Two weeks after the last DNA vaccination, spleen cells from vaccinated animals in various groups were harvested and cultured for 24 h at 37°C in 5% CO₂ with either 20 µg/ml of the extract of TUBO cells (T: test) or (5 µg/ml) PHA (P: positive control) or left untreated (N: negative control). The cells were then pulsed for 48 h with 5-bromo-2'-deoxyuridine (BrdU)-labeling solution. Uptake of BrdU was detected using the cell proliferation ELISA BrdU kit (Roche Diagnostic GmbH, Mannheim, Germany) and expressed as stimulation index (SI):

$$SI = [(T - N)/(P - N)] \times 100$$

CTL assay

The CTL activity of the spleen cells of the tumor-bearing and variously vaccinated mice was assayed immediately by lactate dehydrogenase release (LDH) assay, according to

manufacturer's instructions (Roche Diagnostic GmbH, Mannheim, Germany). Various concentrations of spleen cells with 1×10^4 target TUBO cells were mixed at 100:1, 50:1, and 25:1 effector/target ratios in round-bottom 96-well microtiter plates in triplicate. After 4 h of incubation at 37°C in an atmosphere containing 5% CO₂ followed by centrifugation at 250×g for 10 min, activity of LDH released from the cells to the medium was measured. The OD_{492 nm} of the supernatants was measured with the Universal Microplate Reader. The percent of cytotoxicity was calculated as follow:

$$\% \text{ Cytotoxicity} = \frac{\text{OD}_{\text{test}} - [\text{OD}_{\text{effector spontaneous}} + \text{OD}_{\text{target spontaneous}}]}{\text{OD}_{\text{target maximum}} - \text{OD}_{\text{target spontaneous}}} \times 100$$

OD_{test} is activity of LDH released from co-cultures of target and effector cells. OD_{effector spontaneous} and OD_{target spontaneous} are activity of LDH released by cultures of effector cells and target cells, respectively. OD_{target maximum} is activity of LDH released from target cells lysed by 2% Triron ×100 (Pharmacia, UK).

IFN-γ and IL-4 production

1×10^6 spleen cells of tumor-bearing and variously vaccinated mice were re-suspended in 1 ml of fresh RPMI-1640 containing 10% FBS and antibiotics and cultured at 37°C and 5% CO₂ for 90 h with 20 μg/ml TUBO cell extract in 24-well plates. The plates were then centrifuged at 250×g for 10 min. Supernatants were then collected and frozen at -70°C, until the samples were analyzed to detect presence of interferon-γ (IFN-γ) and interleukin-4 (IL-4) using a DuoSet ELISA system (R&D Systems) according to the manufacturer's instructions.

Flow cytometric analysis of the tumor CD4⁺/CD8⁺ cells and spleen/tumor CD4⁺CD25⁺foxp3⁺ subpopulations

The solid tumors were cut into small pieces, minced with forceps and scalpel, rinsed twice with phosphate-buffered saline (PBS), and passed through a 150-μm stainless steel mesh. The mononuclear cells (MNCs) of the suspension were isolated by density centrifugation (700×g, 15 min, and 20°C) using ficoll hypaque (Sigma, UK). Then, the layer containing MNCs was removed, washed twice with PBS for 10 min in 360×g and 4°C, and the precipitated cells were re-suspended in PBS containing 2% fetal calf serum (Gibco, England). The viability of the cells was determined using tripan-blue (90%); 1×10^5 of the cells were labeled for CD4⁺ and CD8⁺ markers using FITC-conjugated anti-CD4⁺ or anti-CD8⁺ monoclonal antibodies or a fluorescein-conjugated anti-mouse IgG2a, as negative control (Becton Dickinson). Flow cytometric analysis was performed with a FACSCalibur (BD Bioscience).

Spleen and tumor MNCs were labeled for CD4⁺, CD25⁺, and foxp3⁺ markers using Mouse Regulatory T Cells kit, according to manufacturer's instructions (eBioscience, UK). The cells were fixed by 4% para-formaldehyde. Three-color flow cytometric analysis was employed with a FACS Calibur (BD Bioscience), and results were analyzed using WinMID 2.9 software (<http://scripps.edu/software.html>; Scripps Institute, La Jolla, CA).

Statistics

The significance of statistical comparisons was calculated using one-way ANOVA and two-tailed Student's *t* test. Values of *P* < 0.05 were considered to represent statistically significant differences. Experiments were performed in triplicate for each mouse. Results are displayed as means ± standard deviations.

Results

Effect of DNA vaccines on established TUBO tumors

There is less than 6% difference of the amino residues between rat Her2/neu and its ortholog in mice (Nagata et al. 1997). TUBO cells grew progressively in BALB/c mice, as they are weakly immunogenic, and the animal does not react against Her2/neu expressed by tumor cells. The tumor growth does not differ from those observed in BALB-neuT mice (Rovero et al. 2000). In this study, we assessed the effect of DNA vaccination with pHer2/CT on the progression of established TUBO tumor in BALB/c mice, comparing to pHer2- or pCT/Her2-vaccinated animals. The protocol of the study is shown in Fig. 1. We observed control of tumor progression in mice vaccinated with pHer2, pHer2/CT, and pCT/Her2 (Fig. 2). Rate of tumor growth was significantly different (*P* < 0.05) in the group vaccinated with pHer2/CT, in comparison with the other groups. The efficacy of DNA vaccination appeared to be in order of pHer2/CT > pCT/Her2 > pHer2. Although, survival rate in both groups (pHer2/CT and pCT/Her2) were the same with no death during the study, 1/5th of the pHer2/CT-treated mice was tumor-free on day 28 until the mice were killed.

Cellular reactivity

The enhancement of the cell-mediated mechanisms associated with the administration of pHer2/CT was evaluated using in vitro proliferation test, CTL assay, and IFN-γ/IL-4 release titration and compared with that of pHer2 or pCT/Her2. Spleen cells were collected 40 days after tumor implantation and re-stimulated with the TUBO cell-raised tumor extract. The proliferative response of spleen cells

Fig. 1 Protocol of DNA vaccination. Thirteen days after tumor implantation, the animals were injected with DNA vaccine intramuscularly three times at weekly intervals. Two weeks after the last vaccination, immunological evaluation was performed

(Fig. 3) from mice vaccinated with pHer2/CT was markedly enhanced as compared to that of spleen cells from pCT/Her2-vaccinated mice ($P=0.01$). The proliferative response of spleen cells appeared to be in order of pHer2/CT>pCT/Her2>pHer2.

The cytotoxicity of spleen cells against TUBO cells was elicited in mice receiving pHer2/CT and pCT/Her2-vaccinated animals (Fig. 4). The cytotoxicity of spleen cells from mice vaccinated with pHer2/CT was markedly enhanced as compared to that of spleen cells from pCT/Her2-vaccinated mice ($P<0.01$).

A significant increase in IFN- γ release was found when spleen cells from animals vaccinated with pHer2/CT plasmid were stimulated with TUBO cell-raised tumor extract in comparison with the saline, empty vector, and pHer2-vaccinated groups. Vaccination with pHer2 did not induce significantly different IFN- γ response in comparison to negative controls. There was no significant difference in IFN- γ release from spleen cells of pHer2/CT-vaccinated animals comparing to pCT/Her2-vaccinated animals (Fig. 5).

In contrast, spleen cells from animals vaccinated with pHer2/CT released significantly higher titers of IL-4, in

comparison to pHer2- ($P<0.001$) or pCT/Her2-vaccinated groups ($P=0.1$).

Spleen and tumor $CD4^+$ $CD25^+$ Foxp3 $^+$ regulatory T cells and tumor $CD4^+$ and $CD8^+$ T cell levels

No differences in the percentage of $CD4^+$ $CD25^{\text{high}}$ Foxp3 $^+$ Tregs in the spleen of the pHer2- or pCT/Her2-vaccinated groups were found, in comparison with the control groups. However, a marginal decline in Tregs percentage at the spleen of pHer2/CT-vaccinated animals was found ($P=0.1$), in comparison with other groups. In contrast, a statistically significant decline in Tregs percentage at the tumor site was found in pHer2/CT-vaccinated mice, in comparison with the animals vaccinated with pCT/Her2 ($P=0.05$). The therapeutic effect appeared to be also Treg level-dependent, thereby demonstrating that Tregs percentage is decreased in the course of vaccination, consistent with better control of the progression of established tumor (Fig. 6a).

Determination of $CD4^+$ / $CD8^+$ at the tumor site revealed a significant increase in $CD4^+$ percentage in mice treated with pHer2/CT ($P<0.001$), in comparison with the other groups. A significant increase in $CD8^+$ cells percentage were observed in pHer2/CT-vaccinated animals ($P<0.001$), in comparison with the saline, empty vector, and pHer2-vaccinated groups (Fig. 6b). We found a reverse correlation between $CD4^+$ and Tregs percentage (Fig. 6c).

Fig. 2 Rate of tumor growth registered from 1 day before beginning of vaccination until 2 weeks after the last vaccination. Tumor masses were measured every other day with calipers in the two perpendicular diameters

Fig. 3 Proliferative response. Spleen cells were re-stimulated with the TUBO cell-raised tumor extract in the presence of BrdU. Results are shown as SI and represent the mean $\pm$ SD

Fig. 4 CTL assay. Each group of mice ($n=5$) were vaccinated three times with saline (filled diamonds), empty vector (open squares), pHer2 (open triangles), or pHer2/CT (ex symbols). Spleen cells were collected 2 weeks after the last vaccination, co-cultured with target TUBO cells at 25:1, 50:1, and 100:1 effector/target ratios, and incubated for 4 h at 37°C with 5% CO₂. Cytotoxicity was measured by LDH release assay

Discussion

Gp96 is a member of HSPs with similar biological characteristics to other chaperones. It has a main role in both the innate and adaptive immune systems (Srivastava 2002). Gp96 could cause secretion of pro-inflammatory cytokine, such as IL-12 and GM-CSF, and maturation of APCs (Binder et al. 2004). Many studies used fusion technology to improve efficiency of Her2/neu DNA vaccination. However, it should be considered that fusion direction could affect efficiency of Her2 DNA vaccination.

In this study, we investigated how different fusion strategy could affect adjuvant activity of gp96 C-terminal domain and efficiency of Her2/neu DNA vaccination. The resultant immune response could not be due to endotoxin contamination, as firstly, we used the PEG method for purification of the constructs (Sambrook and Russell 2001), which is believed to be most suitable method for endotoxin removal and DNA immunization studies (Boyle et al. 1998). Secondly, endotoxin contamination should have induced a nonspecific immune response with all constructs equally and the GP96 C-terminal-minus Her2/neu construct in particular. Thirdly, it has been demonstrated that effect of endotoxin on immune response is route-dependent (Boyle et al. 1998).

CD8⁺ lymphocytes have an important role in the eradication of an established and actively growing carcinoma (Curcio et al. 2003). Consistently, our results showed a similar pattern of increase in CD8⁺ cells at the tumor site of mice vaccinated with pHer2/pCT, although these cells could be either CD8⁺ T cells or CD8⁺ dendritic cells. However, there was no significant difference in CD8⁺ cells raised by pCT/Her2 or pHer2/CT administration.

Since CD8⁺ T cells exert a major immunological role in tumor rejection, we compared in vitro cytotoxic function of the spleen cells from the various vaccinated animals. We observed significant increase in CTL activity of spleen cells from mice treated with pHer2/pCT in comparison with pCT/Her2DNA vaccine. CD4⁺ increase in pHer2/pCT-vaccinated mice was also significantly higher than those vaccinated with pCT/Her2. These CD4⁺ cells could be either CD4⁺ T cells, which provide better and/or more help required for CTL activity, or CD4⁺ macrophages, which also induce CD4⁺ T cell-independent antitumor immunity in mice (Lee et al. 2003).

The role of Tregs was previously addressed showing an increase and prevalence of Tregs in the peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. Accordingly, depletion of Tregs led to a specific immune T-cell response (Nizar et al. 2009). Such a phenomenon was observed in our experiments: a decreased percentage of Tregs was accompanied by an increased percentage of CD8⁺ cells at the tumor sites of pHer2/CT- or pCT/Her2-vaccinated animals. Interestingly, Treg percentage at the tumor site of the animals vaccinated with pHer2/CT was significantly lower than that of pCT/Her2-vaccinated mice.

Considering lower copying activity of Tregs in spleen rather than tumor, Treg change at spleen of saline-, vector-, pHer2-, or pCT/Her2-vaccinated animals was not detectable (Lutsiak et al. 2008). The point that pHer2/CT vaccination led to Treg decrease in spleen is considerable. It appears that pHer2/CT could profoundly affect percentage of Treg population at the spleen and tumor site of the vaccinated animals, in comparison with pCT/Her2. DNA vaccination using pHer2/CT could act more potent than pCT/Her2 and

Fig. 5 IFN-γ and IL-4 responses in DNA-vaccinated mice. The level of responses was determined by a DuoSet R&D ELISA system after 2 weeks of the last immunization. Spleen cells were stimulated with TUBO cell-raised tumor extract in RPMI 1640 10% FBS for 90 h. Vaccination with pHer2/CT or pCT/Her2 led to a significant increase in IFN-γ level (black columns) and IL-4 secretion (white columns)

Fig. 6 Freshly isolated spleen cells and tumor MNCs were stained with anti-CD4⁺ FITC, anti-CD25 PE, and anti-foxp3 PECy5. After gating on CD4⁺CD25⁺ cells, Tregs were defined as CD25^{high} and foxp3⁺ (a). Tumor MNCs were labeled with FITC conjugated anti-

CD4⁺ or -CD8⁺, freshly (b). We found a reverse correlation between Tregs and CD4⁺ percentage. As Tregs decrease, CD4⁺ cells increase at the tumor site (c)

likely mediate decreased expression of some of the molecules, which usually are expressed in the chaotic microenvironment of tumor and interfere with factors that signal cell survival/death to Tregs. Also, DNA vaccination using pHer2/CT could likely stimulate fibrotic reaction, mediated by decreased survival signals transferred to the tumor cell, more potent than pCT/Her2 DNA vaccine. In addition, it has been reported that Tregs exert their inhibitory effect on CD8⁺ T cells by blocking CD4⁺ T cell help (Chaput et al. 2007). Thus, pHer2/CT DNA vaccine affects Treg cell percentage from one hand and enhances cytotoxic activity by CD8⁺ T cells on the other hand as mentioned above.

As mentioned above, there was an increase in CTL activity and CD8⁺ cells at the tumor site of mice vaccinated with pHer2/pCT, which could be CD8⁺ dendritic cells. Gp96 is a potent inducer of dendritic cell maturation. It has been suggested that continuous and potent stimulation of dendritic cells is needed to break the suppression mediated by Tregs and maturation of dendritic cells alone was not

sufficient (Yang et al. 2004). Our result is in favor of the report suggesting interaction between gp96 C-terminal domain and TLR2, 4 on dendritic cells (Hemmi and Akira 2005; Liu et al. 2009). Presumably, pHer2/CT vaccination could exert its effect on Tregs in two ways: (1) continuous and potent stimulation of dendritic cells to break Tregs-mediated suppression of immune response; (2) decrease of spleen and tumor Tregs thereby, relieving CD4⁺ T cells to provide better help for CD8⁺ T cells.

It has been shown that tumor rejection in established tumor model was dramatically abrogated in the absence of IFN- γ (Curcio et al. 2003). Consistently, our results showed that IFN- γ level was significantly increased in pHer2/CT- or pCT/Her2-vaccinated animals, in comparison with the control groups. However, there was no significant difference on IFN- γ level produced by pHer2/CT- or pCT/Her2-vaccinated animals. Therefore, the difference between the two vaccines is not due to IFN- γ level. The pattern of IL-4 level change was different. The animals vaccinated with pHer2/CT showed a significant raise at the IL-4 level,

in comparison with pCT/Her2-vaccinated mice. Increase of IL-4 level in the animals vaccinated with pHer2/CT or pCT/Her2 is consistent with the previous results demonstrating the dependency on IL-4 in Her2/neu tumor rejection (Amici et al. 1998; Rovero et al. 2000). Such dependency on IL-4 may be related to previous results demonstrating that cytotoxic and Th1 antitumor immunity required IL-4 during the priming phase (Schuler et al. 1999). Effect of IL-4 increase in pHer2/CT-vaccinated animals could also be seen from another view. As mentioned above, significant increase of CD4⁺ cells at the tumor site of pHer2/CT-vaccinated animals was observed. These CD4⁺ cells could also include macrophages. Based on previous report, demonstrating enhanced antibacterial potentials of macrophages mediated by induction of leukocyte infiltration by IL-4 (Ratthé et al. 2009; Wirth et al. 1989), it could be concluded that increase in IL-4 led to enhanced antitumor potency of macrophages at the tumor site. It could greater stress the antitumor potential of IL-4 (Amici et al. 1998). Moreover, increased IFN- γ /IL-4 level in the pHer2/CT-vaccinated animals brings up the involvement of specific antibodies. The important role of antibodies in dealing with the eradication of pre-established tumors has been previously shown (Curcio et al. 2003). We did not assay antibodies raised in our setting, but we do not exclude their role in the control of tumor growth.

N-terminally fusion of Her2/neu to gp96 C-terminal domain might have caused conformational change or steric hindrance, which resulted in the decrease of Her/neu DNA vaccine efficiency, consistent with previous report (Pakravan et al. 2010b). It was previously shown that the signal sequence at the beginning of Her2 sequence have critical role in the potency of Her2 DNA vaccine (Wei et al. 1999). Our results propose that N-terminally fusion of gp96 C-terminal domain to Her2/neu decreased the effect of the signal sequence. Moreover, it has been demonstrated that 70 N-terminal residues of Her2/neu play important role in effective protection against Her2-expressing tumors (Rolla et al. 2008). Therefore, it is also likely that the effect of N-terminal residues of Her2/neu is affected by fusion of gp96 C-terminal domain. On the other hand, different fusion direction might have induced different pattern of ubiquitination on the product of each construct, i.e., pCT/Her2 or pHer2/CT (reviewed by Hoeller and Dikic 2009).

Collectively, our results show that pHer2/CT is able to increase IFN- γ /IL-4 secretion and CD4⁺ cells at the tumor site, which could include macrophages as well as specific immune T-cell response. In addition, significant increase of IL-4 by pHer2/CT vaccination is consistent with antitumor potential of IL-4. Such suggestion is in parallel with previous report on antibacterial role of IL-4 (Wirth et al. 1989). Vaccination with pHer2/CT also led to CD8⁺ cell proliferation at the tumor site along with an increase in the

percentage of specific cell lyses (CTL activity). It could greater stress the pivotal role of Tregs in cancer therapy, as higher CTL activity and decline in Tregs were observed at spleen and tumor site of pHer2/CT-vaccinated mice, in comparison with pCT/Her2-vaccinated group. All these characteristics point to the potent adjuvant activities of gp96 C-terminal domain when fused downstream of Her2/neu and brings up significance of fusion direction in DNA vaccine and fusion technology. Several unanswered questions remain to be elucidated, including conformational changes and different ubiquitination pattern in pHer2/CT, which increase efficiency of the vaccine in comparison with pCT/Her2. More research is needed to *illucidate* the underlying mechanism.

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Declaration of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Amici A, Venzani FM, Concetti A (1998) Genetic immunization against neu/erbB2 transgenic breast cancer. *Cancer Immunol Immunother* 47:183–190
- Binder RJ, Vatner R, Srivastava P (2004) The heat-shock protein receptors: some answers and more questions. *Tissue Antigens* 64:442–451
- Boyle JS, Brady JL, Koniaras C, Lew AM (1998) Inhibitory effect of lipopolysaccharide on immune response after DNA immunization is route dependent. *DNA Cell Biol* 17(4):343–348
- Chaput N, Darrasse-Jèze G, Bergot AS, Cordier C, Ngo-Abdalla S, Klatzmann D et al (2007) Regulatory T cells prevent CD8 T cell maturation by inhibiting CD4 Th cells at tumor sites. *J Immunol* 179:4969–4978
- Curcio C, Di Carlo E, Clynes R, Smyth MJ, Boggio K, Quaglini E et al (2003) Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. *J Clin Invest* 111:1161–1170
- Hemmi H, Akira S (2005) TLR signalling and the function of dendritic cells. *Chem Immunol Allergy* 86:120–135
- Hoeller D, Dikic I (2009) Review article targeting the ubiquitin system in cancer therapy. *Nature* 458:438–444
- Ishikawa T, Kobayashi M, Mai M, Suzuki T, Ooi A (1997) Amplification of the c-erbB-2 (Her-2/neu) gene in gastric cancer cells. *Am J Pathol* 151:761–768
- Kim JH, Majumder N, Lin H, Chen J, Falo LD Jr, You Z (2005) Enhanced immunity by NeuEDhsp70 DNA vaccine is needed to combat an aggressive spontaneous metastatic breast cancer. *Mol Ther* 11:941–949
- Lee JM, Merritt RE, Mahtabifard A, Yamada R, Kikuchi T, Crystal RG et al (2003) Intratumoral expression of macrophage-derived chemokine induces CD4⁺ T cell-independent antitumor immunity in mice. *J Immunother* 26:117–129

- Lin CC, Chou CW, Shiau AL, Tu CF, Ko TM, Chen YL et al (2004) Therapeutic HER2/Neu DNA vaccine inhibits mouse tumor naturally overexpressing endogenous neu. *Mol Ther* 10:290–301
- Liu Z, Li X, Qiu L, Zhang X, Chen L, Cao S et al (2009) Treg suppress CTL responses upon immunization with HSP gp96. *Eur J Immunol* 39:3110–3120
- Lutsiak ME, Tagaya Y, Adams AJ, Schlom J, Sabzevari H (2008) Tumor-induced impairment of TCR signaling results in compromised functionality of tumor-infiltrating regulatory T cells. *Immunol* 180:5871–5881
- Nagata Y, Furugen R, Hiasa A, Ikeda H, Ohta N, Furukawa K et al (1997) Peptides derived from a wild-type murine protooncogene c-erbB-2/HER2/2 neu can induce CTL and tumor suppression in syngeneic hosts. *J Immunol* 159:1336–1343
- Nizar S, Copier J, Meyer B, Bodman-Smith M, Galustian C, Kumar D et al (2009) T-regulatory cell modulation: the future of cancer immunotherapy? *Br J Cancer* 100:1697–1703
- Orlandi F, Venzani FM, Concetti A, Yamauchi H, Tiwari S, Norton L et al (2007) Antibody and CD8+ T cell responses against HER2/neu required for tumor eradication after DNA immunization with a Flt-3 ligand fusion vaccine. *Clin Cancer Res* 13:6195–6203
- Pakravan N, Soleimanjahi H, Hassan ZM (2010a) GP96 C-terminal improves Her2/neu DNA vaccine. *J Gene Med* 12:345–353
- Pakravan N, Soudi S, Hassan ZM (2010b) N-terminally fusion of Her2/neu to HSP70 decreases efficiency of Her2/neu DNA vaccine. *Cell Stress Chaperones* 15(5):631–638
- Pakravan N, Langroudi L, Hajimoradi M, Hassan ZM (2010c) Co-administration of GP96 and Her2/neu DNA vaccine in a Her2 breast cancer model. *Cell Stress Chaperones* June 12, in press
- Quaglino E, Mastini C, Iezzi M, Forni G, Musiani P, Klapper LN et al (2005) The adjuvant activity of BAT antibody enables DNA vaccination to inhibit the progression of established autochthonous Her-2/neu carcinomas in BALB/c mice. *Vaccine* 23:3280–3287
- Ratthé C, Ennaciri J, Garcês Gonçalves DM, Chiasson S, Girard D (2009) Interleukin (IL)-4 induces leukocyte infiltration in vivo by an indirect mechanism. *Mediators Inflamm* 2009:1–10
- Rolla S, Marchini C, Malinarich S, Quaglino E, Lanzardo S, Montani M et al (2008) Protective immunity against neu-positive carcinomas elicited by electroporation of plasmids encoding decreasing fragments of rat neu extracellular domain. *Hum Gene Ther* 19(3):229–240
- Rovero S, Amici A, Carlo ED, Bei R, Nanni P, Quaglino E et al (2000) DNA vaccination against rat Her-2/neu p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* 165:5133–5142
- Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 138–161
- Schuler T, Qin Z, Ibe S, Noben-Trauth N, Blankenstein T (1999) T helper cell type 1-associated and cytotoxic T lymphocyte-mediated tumor immunity is impaired in interleukin 4-deficient mice. *J Exp Med* 189:803–810
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE et al (1989) Studies of the Her-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244:707–712
- Srivastava P (2002) Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. *Annu Rev Immunol* 20:395–425
- Wei WZ, Shi WP, Galy A, Lichlyter D, Hernandez S, Groner B et al (1999) Protection against mammary tumor growth by vaccination with full length, modified human ErbB-2 DNA. *Int J Cancer* 81:748–754
- Wirth JJ, Kierszenbaum F, Zlotnik A (1989) Effects of IL-4 on macrophage functions: increased uptake and killing of a protozoan parasite (*Trypanosoma cruzi*). *Immunol* 66:296–301
- Yang Y, Huang CT, Huang X, Pardoll DM (2004) Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol* 5:508–515