Comparison of adjuvant activity of N- and C-terminal domain of gp96 in a Her2-positive breast cancer model

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Abstract It has been frequently reported that gp96 acts as a strong biologic adjuvant. Some studies have even investigated adjuvant activity of the gp96 C- or N-terminal domain. The controversy surrounding adjuvant activity of gp96 terminal domains prompted us to compare adjuvant activity of gp96 C- or N-terminal domain toward Her2/neu, as DNA vaccine in a Her2/neu-positive breast cancer model. To do so, mice were immunized with DNA vaccine consisting of transmembrane and extracellular domain (TM + ECD) of rat Her2/neu alone or fused to N- or C-terminal domain of gp96. Treatment with Her2/neu fused to N-terminal domain of gp96 resulted in tumor progression, compared to the groups vaccinated with pCT/Her2 or pHer2. Immunological examination revealed that treatment with Her2/neu fused to N-terminal domain of gp96 led to significantly lower survival rates, higher interferon- γ secretion, and induced infiltration of CD4⁺/CD8⁺ cells to the tumor site. However, it could not induce cytotoxic T lymphocyte activity, did not decrease regulatory T cell percentage at the tumor site, and eventually led to tumor progression. Our results reveal that gp96 N-terminal domain does not have adjuvant activity toward Her2/neu. It is also proposed that adjuvant activity and the resultant immune response of gp96 terminal domains may be directed by the antigen applied.

Keywords Her2 · gp96 terminal domains · Regulatory T cells · Adjuvant activity · Breast cancer · Tumor progression

Introduction

The Her2/ErbB2/neu proto-oncogene is one of the tumor-associated antigens, regarded as an ideal target of immunotherapy because of its elevated expression in tumors, cell surface localization, and its involvement in cancer progression/worsening prognosis. It is a member of the epidermal growth factor receptor family and is 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 has been shown to have protective and therapeutic potential against Her2/neu-expressing mammary tumors (Lin et al. 2004; Quaglino et al. 2005; Rovero et al. 2000; Wei et al. 1999). New strategies have been applied to enhance both the quality and quantity of the immune response against Her2-expressing tumors. Some studies have used the Her2 gene with cytokine or other molecules such as heat shock proteins (HSPs) involved in regulation of the immune response to enhance the potency of Her2/neu DNA vaccines (Cappello et al. 2003; Chang et al. 2004; Dela Cruz et al. 2005; Disis et al. 2003; Lin et al. 2004; Orlandi et al. 2007; Spadaro et al. 2005; Wei et al. 1999).

gp96 is a member of the HSP90 family and plays important roles in innate and adaptive immune responses, besides protein folding and assembly (Srivastava 2002; Nicchitta 2003). It has been frequently reported that gp96 isolated from tumors or virus-infected cells elicits a specific cytotoxic response against their origins. This is because gp96 is capable of cross-presenting antigenic peptides to the major histocompatibility complex class I pathway of

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antigen-presenting cells (APCs) upon engagement of its receptor on APCs (Binder 2008). However, gp96 remains a mysterious molecule, as issues regarding its peptide-binding site and interaction with toll-like receptors are controversial (reviewed by Nicchitta et al. 2004; Facciponte et al. 2006). Another important issue is the adjuvant activity of gp96 C- and N-terminal domains. Reports on the adjuvant activity of gp96 C-terminal domain are controversial, depending on the antigen applied (Li et al. 2005a; Rapp and Kaufmann 2004). Our previous experiments demonstrated that fusion, but not co-administration, of the gp96 C-terminal domain to Her2/neu led to improved inhibition of tumor growth (Pakravan et al. 2010a). Previously, it was demonstrated that the gp96 N-terminal domain has potent adjuvant activity toward hepatitis B surface antigen (HBsAg; Li et al. 2005b; Yan et al. 2007). In this study, the adjuvant activity of gp96 N- and the C-terminal domain was compared in a Her2 breast cancer model. Mice with established Her2-expressing tumors were vaccinated using DNA vaccine containing the N- or C-terminal domain of gp96 fused to TM + ECD of the rat Her2/neu. The resultant immune response was then evaluated and compared. It should be noted that this study was conducted at the same time of the previous report (Pakravan et al. 2010a). Some of the results on the adjuvant activity of gp96 C-terminal toward TM + ECD of rat Her2/neu were previously published (Pakravan et al. 2010a), and statistical analysis with the gp96 N-terminal fused to TM + ECD of rat Her2/neu has been mentioned here for comparison.

Material and methods

Mice

Eight- to 10-week-old female BALB/c mice were purchased from the 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 the 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 Dulbecco's modified Eagle's medium (CytoGen, Germany)

supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (BioSera Ltd, UK), and 20% heat-inactivated fetal bovine serum (FBS; Gibco BRL) 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 with calipers in the two perpendicular diameters. Vaccination was started 13 days after tumor implantation.

DNA expression vectors and vaccination

TM + ECD of 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). Half downstream or N-terminal of human gp96 (kindly provided by Dr Brian Seed, Harvard Medical School, Boston, MA, USA; GenBank accession no. NM_003299) was cloned into pHer2, upstream of Her2/neu (pNT/Her2) between Hind III/Nar I (Nar I was located just at the beginning of Her2/neu). To ensure rotational freedom of the fusion genes product, a flexible sequence encoding for glycine was located between the N-terminal domain of gp96 and Her2/neu. Construction of pCT/Her2 was described previously (Pakravan et al. 2010a). The expression of each construct was evaluated by the cloning of green fluorescent gene (GFP) at the end of gene (Chalfie et al. 1994; see below). The arrangement of the genes in each construct was as follows:

pNT/Her2/GFP : gp96N – terminal – – – Her2 – – – – – GFP

pCT/Her2/GFP : gp96 C – terminal – – – Her2 – – – – – GFP

All of the constructs were confirmed by sequencing. Therefore, since all the genes are in frame and the stop codon was just located at the end of the GFP gene, expression of GFP is equivalent to the expression of the preceding genes located before the GFP gene.

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

Mice (five in each group with the exception of pNT/Her2-vaccinated group with 12 mice because of low survival rate) were grouped into six cages, and at day 0, the tumor was implanted. Starting 13 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, pNT/Her2, or pCT/Her2. Mice were sacrificed 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 sacrificed.

In vivo gene expression

To confirm that in vivo expression of each construct occurred, gene expression was shown using mouse muscle cells. The shaved point of injection was marked on the muscle, and 25 µg of pNT/Her2/GFP or pCT/Her2/GFP plasmid or pcDNA3* in a volume of 60 µl was administered along the longitudinal axis of the mouse quadriceps muscle and parallel to the myofibers using a 28-gauge needle and a 0.5-cc insulin syringe. Mice were sacrificed 11 days after plasmid DNA injection. The muscles were then removed, frozen in OCT tissue embedding medium, and sectioned (8 µm) with a cryostat. Sections were placed on slides and GFP expression was observed by direct fluorescence microscopy and photographed with a digital camera.

Proliferation assay of spleen cells

An extract of TUBO cells was prepared by making a suspension of a tumor mass, passing it through a mesh, freezing/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 cultured for 24 h at 37°C in 5% CO₂ with either 20 µg/ml of the extract of TUBO cells (test, *T*) or (5 µg/ml) PHA (positive control, *P*) or left untreated (negative control, *N*). 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):

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

Cytotoxic T lymphocytes assay

The CTL activity of the spleen cells of tumor-bearing and variously treated mice was assayed by lactate dehydrogenase release (LDH) assay, according to the manufacturer's instructions (Takara, Japan). Various concentrations of spleen mononuclear 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, the 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 follows:

% Cytotoxicity

$$= \frac{OD_{\text{test}} - [OD_{\text{effector spontaneous}} + OD_{\text{target spontaneous}}]}{OD_{\text{target maximum}} - OD_{\text{target spontaneous}}} \times 100$$

OD_{test} is activity of LDH released from co-cultures of target and effector cells. OD_{effector spontaneous} and OD_{target spontaneous} are activities 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% Triton X-100 (Pharmacia, UK).

Interferon-γ and interleukin-4 production

Spleen cells of 1×10^6 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 the 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 the use of forceps and scalpel, rinsed twice with phosphate buffer 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, UK). The viability of the cells was determined using Trypan blue (90%); 1×10^5 cells were labeled for CD4⁺ and CD8⁺ markers using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 or anti-CD8 monoclonal antibodies or a fluorescein-conjugated anti-mouse IgG2a, as a 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 a Mouse Regulatory T Cells kit,

according to the manufacturer's instructions (eBioscience, UK). The cells were fixed by 4% paraformaldehyde. Three-color flow cytometric analysis was employed with a FACSCalibur (BD Bioscience), and the results were analyzed using WinMID 2.9 software (<http://scripps.edu/software.html>; Scripps Institute, La Jolla, CA, USA).

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. Survival rates were illustrated by Kaplan–Meier plots and compared using the log-rank test.

Results

In vivo gene expression

To investigate gene expression, we used the GFP gene and the cloned GFP gene (Chalfie et al. 1994) before the stop codon located at the end of gp96 N- or C-terminal in pNT/Her2 or pCT/Her2 and named as pNT/Her2/GFP or pCT/Her2/GFP, respectively. Indeed, we attempted the labeling of each gene, so that each protein product of the construct was tagged with green fluorescent protein.

To test whether pNT/Her2/GFP and pCT/Her2/GFP constructs could be expressed in vivo properly, we used mouse muscle cells as an in vivo environment. Constructs of pNT/Her2/GFP, pCT/Her2/GFP, or pcDNA3* were transferred into mouse muscle cells via direct injection. Eleven days later, muscle cells were removed in order to examine the expression of the gene products by direct fluorescence microscopy. Positive gene expression was observed in the cell sections administered by pNT/Her2/GFP or pCT/Her2/GFP (Fig. 1a, b). No fluorescent signal was observed from the control samples using injection of pcDNA3* (data not shown). Fluorescent dye due to NT-Her2-GFP or CT-Her2-GFP protein production in the transfected muscle cells was detected using fluorescence microscopy, confirming the successful construction and correct expression of the constructs in mouse muscle cells. We did not observe significant differences in the microscopic view of each sample. DNA sequence analysis also revealed that the gp96 N- or C-terminal and Her2 gene were located in frame with the GFP gene.

Effect of DNA vaccines on established tumors

There is less than a 6% difference in the amino residues between rat Her2/neu and its surrogate in mice. It can be

Fig. 1 Photograph of in vivo gene expression, 11 days after injection of **a** pNT/Her2/GFP or **b** pCT/Her2/GFP observed by direct fluorescence microscopy

regarded as a xenogeneic resource of TAA (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 (Rovero et al. 2000). In this study, we assessed the effect of DNA vaccination with pNT/Her2 on the progression of established TUBO tumor in BALB/c mice, compared to pHer2- or pCT/Her2-vaccinated animals. The protocol of the study is shown in Fig. 2. We did not observe a control of tumor progression in mice vaccinated with pNT/Her2. In contrast, pHer2 and pCT/Her2 ($P < 0.05$) appeared to control tumor progression more effectively, in comparison to pNT/Her2-vaccinated mice. The efficacy of DNA vaccination appeared to be in order of pCT/Her2 > pHer2 > pNT/Her2, as shown in Fig. 3. It rather seems that fusion of the N-terminal domain of gp96 to TM + ECD of Her2/neu reduces the efficacy of the vaccine, at least when it comes to the effect on tumor growth in vivo.

Fig. 2 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

Survival studies were performed (Fig. 4) to further evaluate the adjuvant activity of gp96 N-terminal domain toward Her2. As shown in Fig. 4, the survival rate of animals vaccinated with pNT/Her2 was significantly different from that of pCT/Her2-vaccinated mice ($P < 0.01$). The mean survival time of mice receiving pCT/Her2 was 20 days after initiation of vaccination. In contrast, the mean survival time of mice receiving pNT/Her2 was 17 days after initiation of vaccination. All these results indicated that gp96 N-terminal decreased therapeutic potentials of Her2 DNA vaccine.

Cellular reactivity

The enhancement of the cell-mediated mechanisms was evaluated using an in vitro proliferation test, CTL assay, and measurement of IFN- γ /IL-4 levels. 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. 5) from mice vaccinated with pNT/Her2 was markedly enhanced as compared to that of spleen cells from pHer2- or pCT/Her2-vaccinated animals ($P < 0.02$).

The cytotoxicity of spleen cells against TUBO cells (target) was not elicited in mice receiving pNT/Her2. Despite this, animals vaccinated with pCT/Her2 displayed cytotoxic response (Pakravan et al. 2010a).

IFN- γ /IL-4 release titration revealed that the animals vaccinated with pNT/Her2 induced a significantly higher IFN- γ level in comparison with a negative control, pHer2-, and pCT/Her2-vaccinated groups ($P < 0.01$), as shown in

Fig. 6. In contrast, IL-4 level was higher in the pNT/Her2-vaccinated group, in comparison with a negative control and the pHer2-vaccinated group ($P < 0.01$).

Spleen and tumor CD4⁺CD25⁺foxp3⁺ regulatory T cells and tumor CD4⁺ and CD8⁺ cell level

No differences in the percentage of CD4⁺CD25^{high}foxp3⁺ regulatory T cells (Tregs) in the spleen of the various treatment groups were found. No statistically significant change in Tregs percentage was found at the tumor site in mice vaccinated with pHer2 or pNT/Her2, in comparison with the control mice. In contrast, a statistically significant decline in Tregs percentage at the tumor site was found in pCT/Her2-vaccinated animals, as discussed previously (Pakravan et al. 2010a). While fusion with the gp96 C-terminal domain gene enhanced the efficacy of Her2 vaccination, fusion with the gp96 N-terminal domain gene decreased the efficacy of Her2 vaccination and had no effect on the CD4⁺CD25⁺foxp3⁺ T cell level at the tumor site (Fig. 7a). In contrast, there was a decrease in Treg/T effector, as shown in Fig. 7b. This indicates that the increase in the Treg/T effector should be due to a T effector cells increase in pNT/Her2-vaccinated animals (consistent with a significant increase in IFN- γ levels) and a decrease in Treg percentage and/or a T effector increase in pCT/Her2-vaccinated animals.

Determination of CD4⁺/CD8⁺ cells percentage at the tumor site revealed a significant increase in the percentage of CD4⁺/CD8⁺ cells in pNT/Her2-vaccinated mice ($P < 0.05$) and pCT/Her2 (Pakravan et al. 2010a), in comparison

Fig. 3 Rate of tumor growth registered from the 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. 4 Survival rate in pNT/Her2- or pCT/Her2-vaccinated animals. Tumor growth was monitored every other day for the duration of the experiments. pNT/Her2 vaccination led to significant shorter survival rate with respect to pCT/Her2-vaccinated mice

with a negative control and pHer2-vaccinated mice (Fig. 7c). While the percentage of CD4⁺ cells in pNT/Her2-vaccinated mice was significantly higher than that of pCT/Her2-vaccinated mice ($P < 0.05$), there was no significant difference in the percentage of CD8⁺ cells between the two groups.

In pCT/Her2-vaccinated mice, an increase in CD4⁺/CD8⁺ cells percentage was accompanied by a decline in Treg percentage, as mentioned above. However, the same phenomenon was not observed for pNT/Her2-vaccinated animals, in which an increase in CD4⁺/CD8⁺ cells percentage was not accompanied by a decline in Treg percentage.

Fig. 5 Proliferative response. Spleen cells were re-stimulated with the TUBO cell-raised tumor extract in the presence of BrdU. Results are shown as percent of SI and represent the mean $\pm$ SD

Fig. 6 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 pNT/Her2 led to a significant increase in IFN- γ level (black columns). In addition, IL-4 level (white columns) was significantly different between pHer2- or pNT/Her2-vaccinated animals. Results are represented as the mean $\pm$ SD

Discussion

gp96 is a member of the HSPs family with similar biological characteristics to other chaperones. It has major effects on both innate and adaptive immune systems (Srivastava 2002) mediated by the induction of DCs maturation and inflammatory cytokine secretion (Binder 2008). While reports on the adjuvant activity of gp96 C-terminal domain are controversial (Li et al. 2005a; Pakravan et al. 2010a; Rapp and Kaufmann 2004), previous studies demonstrated that gp96 N-terminal domain has potent adjuvant activity towards HBsAg (Li et al. 2005b; Yan et al. 2007). In this study, we examined adjuvant activity of the gp96 N-terminal domain and compared it with gp96 C-terminal, which was shown to have adjuvant activity toward Her2 (Pakravan et al. 2010a). To do so, we fused gp96 N- or C-terminal domain to TM + ECD of the rat Her2/neu (pNT/Her2 or pCT/Her2, respectively). Immunological responses triggered by pNT/Her2, when administered intramuscularly to BALB/c mice with established tumors, were then evaluated and compared with that of pCT/Her2 and pHer2.

Our previous results showed that DNA vaccination using pCT/Her2 could slow down progression of the established tumors. Surprisingly, DNA vaccination using fusion of N-terminal domain of gp96 gene to TM + ECD of rat Her2/neu could not further enhance the therapeutic efficacy. Since in vivo gene expression analysis did not show significant difference in gene expression of each construct, the resultant immune response could not be due to different expression of each construct. Hence, the lack of vaccine effectiveness should be analyzed locally and systemically, i.e., systemic effect of vaccine and the tumor–host mutual talk at the tumor foci.

Fig. 7 Freshly isolated spleen cells and tumor MNCs from 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⁺ the percentage of Tregs was determined (**a**). Also ratio of Treg/T effector was determined (**b**). Tumor MNCs were labeled with FITC-conjugated anti-CD4 or anti-CD8, freshly. CD4⁺ percentage was observed to increase significantly in pNT/Her2- and marginal increase in the pHer2-vaccinated animals. Increase at the CD8⁺ percentage to be in the order of pCT/Her2>pNT/Her2>pHer2 (**c**)

From the systemic view, the level of IFN- γ was significantly increased in the pNT/Her2-vaccinated animals, in comparison with those in the pCT/Her2-vaccinated groups. Impotency of pNT/Her2 could be because of elicitation of a different type and strength of immune response. It seems that over-production of IFN- γ by pNT/Her2, in comparison with pCT/Her2, might have led to elicitation of an inefficient and/or inappropriate type of

immune response. Based on previous results, it is likely that there is an optimum level of production for each cytokine (He et al. 2005; Kaplan et al. 1998; Pakravan et al. 2010a, b, c), and the level produced by pNT/Her2 could be considered as over-production or immune pressure (Block and Markovic 2009; Liu et al. 2005) which presumably leads to an unresponsive state to IFN- γ via different mechanisms (Beatty and Paterson 2000; He et al. 2005; Kaplan et al. 1998). It has been suggested that those tumors growing in the face of immune pressure become resistant to immune attack by a variety of mechanisms (reviewed by Block and Markovic 2009). The occurrence of such a phenomenon regarding Her2-positive tumors has been addressed frequently (Kmieciak et al. 2007; Manjili and Kmieciak 2008; Marth et al. 1990; Wang et al. 2008; Worschech et al. 2008).

From local view, significantly higher IFN- γ secretion by pNT/Her2-vaccinated mice might have affected the tumor microenvironment and the expression of molecules in a way that attracts and activates inhibitory immune cells or other robust immune mechanisms which favored tumor growth (Lepique et al. 2009; Shevach 2006; Spoelstra et al. 1999; Thornhill et al. 1991). This has been confirmed by the results on the level of Tregs at the tumor site, which did not decrease in pNT/Her2-vaccinated animals, in comparison with pCT/Her2-vaccinated mice. It is also consistent with the previous report (Nishikawa et al. 2005) demonstrating involvement of IFN- γ in Treg activation. An increase in CD4 cells at the site of tumor might include effector T cells (producing significant IFN- γ levels, as shown in Fig. 7b) leading to increased and/or activation of suppressive T cells, and/or other suppressive immune cells such as tumor-associated macrophages (reflected by an increase in CD4⁺ cells at the tumor site) with an inhibitory effect on the anti-tumor immune response (Lepique et al. 2009; Shevach 2006). CD8⁺ cells could also include suppressor dendritic cells and/or CD8⁺ suppressor T cells which have been shown previously to represent one of the major mechanisms of tumor escape (Shafer-Weaver et al. 2009; Nagaraj and Gabrilovich 2008).

We previously reported that N-terminally fusion of Her2/neu to HSP70 (pHSP70/Her2) decreased the efficiency of Her2/neu DNA vaccine (Pakravan et al. 2010b). Tumor progression and IFN- γ /IL-4 levels were similar to that of pNT/Her2, but pHSP70/Her2 decreased Treg percentage at the tumor site and, importantly, increased survival rate. However, pNT/Her2 did not decrease Treg percentage at the tumor foci. Higher survival rates in the pHSP70/Her2-treated mice was concomitant with a significant decrease in Treg percentage at the tumor site, in comparison with pNT/Her2-treated mice. Considering the critical role of Tregs in pregnancy, it is consistent with our results on rat pregnancy (unpublished data) in which administration of pCT/Her2 to

pregnant rats led to rapid pregnancy loss, whereas administration of pNT/Her2 did not. This suggests a critical role for Treg on survival rates and the quality of life. Moreover, it suggests that unknown elements other than IFN- γ (Nishikawa et al. 2005) may control the activity and/or quantity of Treg cells.

Collectively, vaccination with pNT/Her2 plasmid led to significantly higher IFN- γ /IL-4 secretion, but no in vitro CTL activity, no decrease in Tregs at the tumor site, and lower survival rates compared with vaccination using pCT/Her2 plasmid. Higher levels of IFN- γ in pNT/Her2-vaccinated animals might have activated an inhibitory mechanism against the anti-tumor immune response. For example, vaccination with pNT/Her2 might have attracted/activated potent cells such as suppressive T cells and tumor-associated macrophages which suppress the T cell anti-tumor response. Our results might be an indication of immune pressure. It means that the relationship between the strength and efficiency of the immune response is not mutually exclusive and higher strength does not necessarily guarantee higher efficiency and quality (Wang et al. 2008).

Our results suggest the following:

- In the case of gp96 N-terminal domain, its adjuvant activity seems to be antigen dependent because gp96 N-terminal domain was previously reported to have adjuvant activity toward HBsAg (Li et al. 2005b; Yan et al. 2007). As previously mentioned, this is the case for C-terminal domain. We and others demonstrated that gp96 C-terminal domain has adjuvant activity (Rapp and Kaufmann 2004), while others claimed it has no adjuvant activity (Li et al. 2005a) or, more correctly, that it has “reverse/negative adjuvant activity.” It also seems that the immune response stimulated using gp96 N-terminal domain is directed by the antigen applied.
- Treg decline was not observed and survival rate was decreased when pNT/Her2 was administered and tumor growth control was not observed. It could emphasize the critical role of Tregs in cancer therapy.
- A higher immune response could sometimes act against tumors, via the emergence of aggressive tumor variants. Presumably, the immune response elicited against a tumor can act as a “double-edged sword” acting in favor of the host or the tumor via different mechanisms. More research should be conducted to elucidate the underlying mechanisms exerted by a pNT/Her2 vaccine.

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Conflict of interest All financial and commercial conflicts of interest are disclosed.

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