A therapeutic Her2/neu vaccine targeting dendritic cells preferentially inhibits the growth of low Her2/neu-expressing tumor in HLA-A2 transgenic mice

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Abstract (250 words)

Purpose: E75, a peptide derived from the Her2/neu protein, is the most clinically advanced vaccine approach against breast cancer. In this study, we aimed to optimize the E75 vaccine using a delivery vector targeting dendritic cells – the B-subunit of Shiga toxin (STxB) - and to assess the role of various parameters (Her2/neu expression, combination with trastuzumab) in the efficacy of this cancer vaccine in a relevant preclinical model.

Experimental Design: We compared the differential ability of the free E75 peptide or the STxB-E75 vaccine to elicit CD8+ T cells, and the impact of the vaccine on murine HLA-A2-tumors expressing low or high levels of Her2/neu.

Results: STxB-E75 synergized with GM-CSF and CpG and proved to be more efficient than the free E75 peptide in the induction of multi-functional and high avidity E75-specific anti-CD8+ T cells resulting in a potent tumor protection in HLA-A2 transgenic mice. High expression of HER2/neu inhibited the expression of HLA-class I molecules, leading to a poor recognition of human or murine tumors by E75-specific cytotoxic CD8+ T cells. In line with these results, STxB-E75 preferentially inhibited the growth of HLA-A2 tumors expressing low levels of Her2/neu. Co-administration of anti-Her2/neu mAb potentiated this effect.

Conclusions: STxB-E75 vaccine is a potent candidate to be tested in patients with low Her2/neu-expressing tumors. It could also be indicated in patients expressing high levels of Her2/neu and low intratumoral T cell infiltration to boost the recruitment of T cells a key parameter in the efficacy of anti-Her2/neu mAb therapy.
Statement of translational relevance

The E75 peptide derived from the Her2/neu protein has been developed as vaccine and generated suboptimal results in clinical trials. Using a delivery vector targeting the E75 peptide to dendritic cells, we improved its potency both in terms of induction of CD8+ T cells, and tumor protection in relevant preclinical models. We also demonstrated in a series of human breast cancer cell lines, that high Her2/neu-expressing tumor cells are not efficiently recognized by anti-E75 CD8+ T cells compared to low Her2/neu tumors. These results were also translated and confirmed in in vivo models. Thus, although, Her2/neu is a validated target for various mAb in breast cancer and gastric cancer expressing high levels of this antigen, this study demonstrates that a Her2/neu cancer vaccine may be preferentially indicated in low Her2/neu-expressing tumors. A synergistic effect was also observed when cancer vaccine was combined with anti-Her2/neu mAb treatment.
Introduction
The Her2/neu proto-oncogene, is a validated target in cancer, as various anti-Her2/neu antibodies (trastuzumab, pertuzumab, ado-trastuzumab emtansine...) or tyrosine kinase inhibitors (lapatinib) demonstrated their clinical efficacy in patients with Her2/neu overexpressing breast and gastric cancers. Unfortunately, even following the best combination of Her2/neu-directed treatments and chemotherapy, the progression free survival in patients with metastatic breast cancer is 18.7 months(1).

Intrinsic properties of Her2/neu may explain the clinical efficacy of these approved drugs and the interest to develop other therapeutic approaches against this molecule. Indeed, its overexpression is required to maintain the malignant phenotype of these cancers and tumor escape by downregulation of Her2/neu is thus more difficult to achieve. Cancer stem cells which contribute to tumor metastasis and treatment resistance, express increased Her2/neu levels(2). Other arguments support the development of active immunotherapy such as anti-Her2/neu T cell vaccines to complement and improve the clinical activity of commercially available anti-Her2/neu antibodies. Her2/neu is immunogenic and elicits natural Her2/neu-specific T cell responses in patients overexpressing this antigen (3, 4). Intratumoral T cell infiltration is associated with a good prognosis in these patients (5) and the presence of high levels of tumor-infiltrating lymphocytes (TIL) were associated with increased trastuzumab benefit in Her2/neu+ breast cancer patients (6).

The role of T cells in controlling Her2/neu-expressing tumors was also emphasized by the demonstration that intratumoral injection of human effector T cells genetically modified to express chimeric antigen receptor (CAR) against Her2/neu completely blocked the growth and metastasis of Her2/neu expressing pancreatic adenocarcinoma xenografts in SCID mice (7).

Various preclinical and clinical studies showed that the combination of anti-Her2/neu antibodies and vaccines capable to inducing anti-Her2/neu T cells synergized in promoting
the regression of Her2/neu expressing tumors (8, 9)(10). In mice and humans, anti-Her2/neu antibodies enhanced the induction of Her2/neu-specific CD8 positive T cells and favor Th1 cell infiltration (11, 12)(13, 14). In accordance with these results, it has been reported that effective Her2/neu antibody treatment also requires an adaptive immune response involving CD8^+T cells(13). These data argue for boosting T cell responses against Her2/neu positive breast cancer and various groups clearly demonstrated the ability of Her2/neu cancer vaccines based on dendritic cells, peptides, recombinant proteins, recombinant virus or DNA to induce specific CD4^+ and CD8^+T cell responses in humans (15-17). However, up until now, no Her2/neu vaccine has been approved for the clinics.

E75, a nine amino-acid peptide derived from the extracellular domain of Her2/neu protein (KIFGSLAFL, Her2/neu residues 369-377)(18) is the best studied Her2/neu derived peptide to date. It binds to HLA-A2 and HLA-A3 expressed in 60-75% of the general population and is processed and presented by tumor cells (19, 20). Although the exact immunodominant peptide (Her2/neu 369-377 or Her2/neu 373-382) presented by tumor cells is debated, anti-E75 CD8^+T cells recognized these two epitopes(21).

The E75 peptide-based vaccine (nelipepimut-S) has been shown to be immunogenic in humans (22, 23), but induced suboptimal results in clinical trials designed for the prevention of clinical recurrence in high risk disease-free breast cancer patients (24). Nevertheless, a phase III clinical trial based on the administration of the E75 peptide vaccine combined with GM-CSF is ongoing, making it the most clinically advanced Her2/neu targeting breast cancer vaccine (NCT01479244).

In parallel, recent impressive clinical results using immunomodulators blocking the PD-1-PD-L1 pathway underscores the major role of anti-tumor CD8^+T cells in tumor regression(25). The E75 peptide vaccine elicited specific CD8^+T cells, but their levels were weak and the increase of E75-specific CD8^+T cells after vaccine administration was most often lower than
2 fold (26-28). This CD8+ T cells waned rapidly and required repetitive booster doses (29), as already observed with other Her2/neu peptide based vaccines (30).

We have developed and validated a non replicative vector that targets dendritic cells, the B subunit of the Shiga toxin (STxB). When coupled to various tumor antigens, STxB elicits a strong induction of specific CD8+ T cells, which can be further enhanced by the addition of adjuvants (31, 32). Compared to other vectors, STxB targets the same glycolipid receptor, gb3 which are identical in all species. In addition, we already demonstrated its efficacy to favor cross presentation in human dendritic cells (33). STxB displays intrinsic immunogenicity demonstrated by a weak induction of anti-STxB antibodies, but which did not interfere with repetitive immunization (31).

The aim of this study was to improve and optimize the E75 peptide vaccine using this delivery vector and to assess the role of various parameters (Her2/neu expression, combination with trastuzumab) in the efficacy of the cancer vaccine in a relevant preclinical model.

Material and methods

Mice

Humanized HLA-A2 transgenic (TG) mice were obtained from Dr YC Lone (INSERM U1014, Hopital Paul Brousse, Villejuif, France) and from Dr F. Lemmonier (Institut Pasteur, Paris, France).

To set up an hHer2/neu-HLA-A2 tumor model, we transfected the B16-HLA-A2 melanoma cell line with a cDNA encoding hHer2/neu. A clone co-expressing HLA-A2 and hHer2 was selected (Supplementary Fig 1A). We then s.c grafted this B16-HLA-A2-hHer2/neu clone and
monitored the tumor growth in HLA-A2 TG mice. Steady tumor growth was reproducibly achieved using at least $5 \times 10^5$ cells (Supplementary Fig 1B).

All mice were kept under specific pathogen–free conditions at the INSERM U970 animal facility. All experiments have been reviewed and approved by Paris-Descartes Ethical Committee for Animal Experimentation (CEEA34.ET.154.12.)

**Cell lines**

B16-A2 cells were obtained from Dr YC Lone (INSERM U1014). B16-A2-Her2/neu cells were generated by stable transfection after electroporation with a plasmid encoding human Her2/neu provided by P. Bruhns (Institut Pasteur) and selected with 0.5 μg/ml of G418 (Invivogen) and 7 μg/ml of blasticidin (Invivogen) supplemented medium.

To obtain the low and high Her2/neu expressing B16-A2 cells, stable B16-A2-Her2/neu transfectants were sorted based on the Her2/neu expression.

SKBR3-A2 breast tumor cells were obtained from T. Dubois (Institut Curie) and UACC812 cells was bought at ATCC (American Type Culture Collection) (Manassas VA, USA). SK-Mel 37 melanoma cells and MCF-7 breast tumor cells were obtained from B. Maillere (CEA, Saclay).

For Elispot experiments, tumors cells lines were first cultured 48h with 100U/ml of rhIFNγ (Peprotech) or Universal IFN type I (R&D system), then treated with 1 μg/ml Mytomycin C for 1h. After washes, cells were used as stimulatory cells in IFNγ Elispot assay (5000 cells/well).

**Flow cytometry**
The anti-mouse CD8 (clone 53-6.7) and anti-human Her2/neu (CD340, clone 24D2) mAb were purchased at Biolegend (Distributor Ozyme, Saint Quentin Velaines, France) and the anti-human HLA-A2 (clone BB7.2) at BD Biosciences (Le Pont de Claix, France). The HLA-A2-Her2/neu369-377 dextramer was obtained from Immudex (Copenhagen, Denmark). For dextramer analysis, cells were incubated with PE-labeled dextramer (45 min at 4°C in the dark). After incubation and washes, labeled anti-CD8 mAbs were used to phenotype the positive dextramer CD8^+T cells. An irrelevant dextramer was used in each experiment to assure the specificity of the reaction against Her2/neu. The background values obtained with cells labelled with the irrelevant dextramer were deduced from the percentage shown.

Acquisitions were performed on BD LSRII, and data were analysis with FlowJo Software (Tree Star).

**Elispot**

CD8^+ splenic T cells were isolated from immunized HLA-A2 TG mice by magnetic beads (Miltenyi Biotec, Paris).

Specific HLA-A2-Her2/neu369-377 CD8^+T cells were sorted by cell sorter (Aria, Becton Dickinson) after dextramer staining.

IFNγ ELISpot kits were purchased at Diaclone (Besançon, France) and used according to the manufacturer’s recommendations. Briefly, in ELISpot IFN-γ mAb coated plates, CD8^+T cells were cultured in duplicate with antigen-presenting cells (APCs) pulsed with the E75 peptide (10 µg/mL) or with tumor cells (after mytomicin C treatment and pre-stimulated or not with rhIFNγ or type I IFN). Plates were cultured for 18h at 37 °C, and spots were revealed following the manufacturer's instructions. Positive controls included cells stimulated with 100ng/ml of phorbolmyristate acetate (PMA) and 500ng/ml of ionomycin (Sigma Aldrich).
Negative controls included cells cultured in the absence of the E75 peptide. IFNγ spot-forming cells were counted on a C.T.L-ELISpot system (C.T.L Bonn. Germany). A response was considered positive if the number of spots in the well stimulated with specific peptide was 2-fold higher than the number of spots in the well without peptide, with a cut-off at 10 spot-forming cells.

**Luminex, In vivo Cytotoxicity**

These techniques are detailed in supplementary data.

**In vivo tumor protection**

In the therapeutic setting, HLA-A2 TG mice were s.c injected with 1.5x10⁶ B16-HLA-A2-Her2/neu tumor cells in the right flank. Once tumors were palpable (around 3-4 days after challenge), mice were vaccinated by the s.c route with the STxB-E75 (0.5nmol) or E75 peptide (0.5nmol) combined with GM-CSF (20 μg). One day later, CpG (50 μg) was s.c injected at the same site. Seven days after the first immunization, mice were boosted with the vaccine alone.

In the prophylactic setting, mice were firstly immunized with the vaccine at day 0 and day 14 and one week later grafted with the tumor cells.

Tumor sizes were measured twice per week by using a caliper (mm² = length x width). For the survival analysis, a tumor size of at least 300 mm² was defined as the experimental endpoint.

For *in vivo* experiment with anti-Her2/neu mAb (4D5, Bioinvest Int), 100 μg of antibody were injected intraperitoneally at the same day as the vaccine.

For *in vivo* CD8⁺T cell depletion, 100 μg of anti-CD8 mAb (YTS 1694, Proteogenix) was injected intraperitoneally once per week, beginning 2 days before the first immunization dose.

**Statistical analyses**
Statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc.). Data were expressed as means ± SD and are representative of at least two independent experiments. Significance was assessed with the Mann-Whitney test to compare two different groups, and Kaplan-Meier curves to compare the survival of the different groups of mice.

Results

1) STxB-E75 vaccine elicits functional T cell responses in HLA-A2 TG mice.

1.1 STxB-E75 synergizes with GM-CSF and CpG for the induction of antigen-specific CD8⁺ T cells.

We have previously demonstrated a synergistic effect between the STxB delivery system and the aGalCer adjuvant (31), which binds to CD1d. Since HLA-A2 TG mice did not express CD1d, we had to evaluate new adjuvants in combination with STxB. We showed that HLA-A2 TG mice immunized with STxB-E75 alone did not elicit a significant anti-E75 CD8⁺ T cell response, as detected by dextramer staining (< 0.1%) or Elispot (Supplementary Fig 2 and data not shown). In contrast, 0.2% or 0.75% of anti-E75 CD8⁺ T cell were detected, when STxB-E75 was co-administered with GM-CSF or CpG at the prime dose respectively (Supplementary Fig 2). Interestingly, a synergy for the induction of anti-E75 CD8⁺ T cell was demonstrated, when GM-CSF and CpG were combined during the prime with CpG given 24 hours after the vaccine and GM-CSF administration (Supplementary Fig 2). Indeed, more than 5% of E75-specific CD8⁺ T cells could be observed with the vaccine system comprising STxB-E75 plus GM-CSF and CpG (Supplementary Fig 2).

1.2 STxB-E75 is more efficient than the non vectorized E75 peptide to elicit functional CD8⁺ T cells.
HLA-A2 TG mice (n = 4) were immunized with 0.5 nmol (20 μg) of STxB-E75, or with 0.5 nmol (3 μg) of the E75 derived peptide, as the optimal 9 mer peptide (Her2/neu369-377), or the optimal 9 mer peptide plus the spacer sequence known to favor cleavage (RRAR), also present as a spacer between the Her2/neu369-377 peptide and STxB in the STxB-E75 protein. All the vaccines were combined with GM-CSF and CpG. Immunization of mice with the E75 peptide alone or the E75 peptide flanked with the RRAR sequence did not induce anti-E75-CD8⁺T cells, as detected by dextramer analysis (Fig 1A) or Elispot (Fig 1C). In contrast, vaccination of mice with the STxB-E75 fusion protein at the same molarity (0.5 nmol) elicited high levels of dextramer positive cells (more than 5%) (Fig 1A and B) and significant levels of IFNγ producing CD8⁺T cells (Fig 1C).

To test for the ability of the E75 peptide to induce specific CD8⁺T cell responses, mice were immunized with 100 μg (25 nmol) of the E75 peptide combined with GM-CSF and CpG, which represents a 50-fold molar excess over the dose used for STxB-E75. Under these conditions, an induction of anti-E75 CD8⁺T cells, reaching a mean value of 0.5% of total CD8⁺T cells, could be observed (Fig 1A) and they produce IFNγ (Fig 1C). However, their levels were much lower than those observed in mice immunized with the STxB-E75 vaccine administered at only 0.5 nmol (Fig 1).

### 1.3 Characterization of the anti-E75 specific CD8⁺T cells induced by E75 or STxB-E75

#### 1.3.1 Multifunctionality of anti-E75 specific CD8⁺T cells

It is well recognized that the quality of CD8⁺T cell response influences the clinical efficacy of anti-tumor vaccine. More specifically, the presence of multifunctional CD8⁺T cells is considered as a relevant read-out to predict a positive clinical outcome (34). The E75 peptide vaccine was used at 50 fold higher molar concentrations than STxB-E75, as no induction of specific CD8⁺T cells was observed at low E75 peptide concentrations (Fig 1). The anti-E75
CD8⁺T cells detected in mice immunized with STxB-E75 produced more chemokines (Rantes, MIP1α) than those induced by the E75 peptide, but we could not conclude from these results about the physiological consequence of these differences (Fig 2A). Moreover, except for the production of IL-13, anti-E75 CD8⁺T cells induced by STxB-E75 or E75 did not produce significant levels (> 10 pg/ml) of the main Th2 (IL-4, IL-5) or Th17 cytokines (IL-17) (Fig 2A). Interestingly, regardless of the vaccine formulation used (E75 peptide or STxB-E75), E75-specific CD8⁺T cells also produced a panel of chemokines (i.e MIP1β, Rantes, eotaxin, IL-9) that play a role in the recruitment of myeloid and T cells, which could amplify locally the anti-tumor immune response (Fig 2A and data not shown). Therefore, the anti-E75 CD8⁺T cells induced by STxB-E75 or E75 are bona fide multifunctional T cells.

We next evaluated the cytotoxic activity of E75-specific CD8⁺T cells elicited in mice immunized with the different vaccine formulations, since this feature is considered a major hallmark of anti-tumor CD8⁺T cells. We demonstrated that the in vivo cytotoxicity effect of anti-E75 CD8⁺T cells induced by STxB-E75 was higher (53% ± 20%) than that elicited by the E75 peptide (12% ± 15%)(Fig 2B)

1.3.2 High avidity of anti-E75 specific CD8⁺T cells elicited by STxB-E75

Various groups reported that the avidity of CD8⁺T cells is an important parameter for their anti-tumor activity (35). We therefore assessed the avidity of the anti-E75 CD8⁺T cells induced after immunization of HLA-A2 TG mice with STxB-E75 or E75. As shown in Fig 2C, the E75 specific CD8⁺T cells induced with STxB-E75 possess a higher avidity than those induced by the E75 peptide. The anti-E75 CD8⁺T cells induced by STxB-E75 immunization can be activated by antigen presenting cells sensitized with as low as 0.001 μg/ml of peptide (Fig 2C).
1.3.3 Persistence of anti-E75 specific CD8⁺T cells elicited by the anti-E75 vaccines

The sustained induction of anti-tumor CD8⁺T cells is a prerequisite for the achievement of a clinical anti-tumor response and the rapid waning of the antigen specific CD8⁺T cells may explain failure of cancer vaccines (29). To address this point, we measured the presence of E75 specific CD8⁺T cells, 30 days after the booster dose administration. At this later time point, the anti-E75 CD8⁺T cells that were elicited by STxB-E75 are present at higher levels and produced more IFNγ than those induced by the E75 peptides (Fig 2D).

2) STxB-E75 is more potent to inhibit the growth of hHer2-HLA-A2 expressing tumors in HLA-A2 TG mice than the E75 peptide.

The efficacy of the STxB-E75 vaccine in controlling hHer2-expressing tumors was tested in both prophylactic and therapeutic setting in HLA-A2 TG mice.

In a therapeutic setting, STxB-E75 clearly delayed tumor growth, when compared to non-immunized mice (p < 0.001), or mice vaccinated with the E75 peptide (p < 0.001)(Fig 3A). In agreement with these results, 50% of mice vaccinated with STxB-E75 were still alive 50 days after the tumor cell engraftment (Fig 3B). Immunization with the E75 peptide alone also had a significant albeit weaker effect on tumor growth, when compared to non-immunized mice (p < 0.05), and 16% of mice immunized with this peptide were alive 50 days after tumor graft (Fig 3B).

In prophylactic experiments, mice were immunized with STxB-E75 or the non-vectorized E75 peptide, both in combination with GM-CSF and CpG. A control group (not vaccinated mice) was also included. One week after the second immunization, mice were grafted with the HLA-A2-hHer2/neu B16 tumor cells. Immunization with STxB-E75 significantly inhibited tumor growth when compared to non-vaccinated mice (p < 0.05) or mice immunized with the E75 peptide (p < 0.01)( Fig 3C). The E75 peptide had no anti-tumor activity when compared
to non immunized mice (Fig 3D). These results were confirmed, when using survival as a read
out. Indeed, 50% of mice vaccinated with STxB-E75 were still alive 40 days after tumor
grafting, whereas all non-vaccinated mice or those immunized with the E75 peptide had died
at day 37 (p < 0.05)(Fig 3D).

To confirm the role of CD8⁺ T cells in the clinical efficacy of the STxB-E75 vaccine, we
demonstrated that the therapeutic tumor protection was lost when the CD8⁺ T cells were
depleted (Fig 3E-F). In addition, after vaccination with STxB-E75, we could detect
intratumoral anti-E75 CD8⁺ T cells (Supplementary Figure 3)

3 Her2/neu expression levels in tumor cells affects the anti-tumor protective efficacy of
the STxB-E75 vaccine

Preclinical results have pointed out the role of Her2/neu expression in the downregulation
of HLA-class I molecules, and preliminary clinical results suggest that the E75 vaccine is less
potent in breast cancer patients whose tumors express high levels of Her2/neu (23, 36). Based
on these evidences, we therefore assessed the ability of anti-E75 CD8⁺ T cells generated by the
STxB-E75 immunization to recognize tumor cell lines expressing variable levels of Her2/neu.
Surprisingly, we found that anti-E75 CD8⁺ T cells were more readily activated by B16-HLA-
A2 tumor cells expressing low levels of Her2/neu, compared to the high Her2/neu HLA-A2
B16 tumors (Fig 4A). In line with these results, human breast tumor cells expressing the
highest levels of Her-2 (SKBR-3 and UACC812) (Supplementary Fig 4B) were not
recognized by the anti-E75 CD8⁺ T cells, whereas the SK-Mel37 melanoma cells and the
MCF-7 breast tumor cells which express moderate levels of Her2/neu were recognized
directly, or in the presence of IFNγ (Fig 4B). IFNγ increases the HLA-A2 expression in the
SKBR-3 breast tumor cells (Supplementary Fig 4), but when co-cultured with the anti-E75
CD8+T cells, the number of spots (n = 6) by the IFNγ Elispot assay remains below the threshold of positivity set at 10 spots (Fig 4).

To build on these results, we sorted B16-HLA-A2-Her2/neulow or B16-HLA-A2-Her2/neu high tumor cells (Supplementary Fig 4). In the therapeutic setting, the STxB-E75 vaccine was more efficient to control the growth of B16-HLA-A2 Her2 low tumors, than the B16-HLA-A2-Her2/neu high tumors (Fig 5A and C). In the survival analysis, 35% of the B16-HLA-A2-Her2/neu low tumor bearing mice treated with the STxB-E75 vaccine were alive at day 50, while none of the B16-HLA-A2-Her2/neu high tumor bearing mice were alive at the same time point (Fig 5B and D). Since a future cancer vaccine targeting Her2/neu has to position considering the current standard therapies, we assessed whether a synergy could be observed between the STxB-E75 vaccine and the anti-Her2/neu mAb. Interestingly, the use of anti-Her2/neu mAb alone was inefficient to inhibit the growth of tumors at any level of Her2/neu expression (Fig 5A and C). In combination with the STxB-E75 vaccine, anti-Her2/neu mAb potentiated the effect of the vaccine on tumor growth in both models, but when survival was analysed, anti-Her2/neu mAb potentiated the efficacy of the vaccine only in the B16-HLA-A2-Her2/neu low tumors, (Fig 5B)

**Discussion**

In this study, we demonstrated that the STxB-E75 vaccine efficiently elicits high levels of long-lasting, multifunctional, cytotoxic and high avidity E75-specific CD8+T cells in HLA-A2 TG mice, which were able to recognize human tumors expressing Her2/neu. Both in terms of concentrations and quality of the induced anti-E75 CD8+T cells, the potency of the STxB-E75 vaccine was far greater than that of the E75 peptide alone, which is currently tested in phase III clinical trials in patients with breast cancer. This difference persisted even when the E75
peptide was used in 50-fold molar excess. This weak immunogenicity of E75 in preclinical model was already reported which supports our results (37).

The Peoples group has been able to correlate the magnitude of the E75-specific T cell responses that were elicited by the E75 vaccine with clinical benefit (23). This observation suggests that the higher potency of the STxB-E75 vaccine to elicit antigen-specific CD8⁺T cells, may improve the clinical response. To support this possible extrapolation, we could show in a preclinical HLA-A2-hHer2/neu tumor model, that the efficacy of STxB-E75 was significantly higher than that of non-vectorized E75 peptide in its capacity to inhibit tumor growth and to improve overall survival. In previous clinical trials, GM-CSF was used in combination with E75 peptide (36). We found that the addition of CpG to GM-CSF greatly improved the magnitude of the anti-E75 CD8⁺T cells induced by STxB-E75, while this effect was less pronounced for the E75 peptide vaccine, similar to what has previously been reported for other peptide vaccines (38). The use of GM-CSF and CpG to recruit dendritic cells and induce maturation of dendritic cells, respectively, may be particularly useful for delivery vectors targeting dendritic cells such as STxB or anti-Dec205 (39).

In order to better position the E75 based vaccine from the clinical perspective, we showed that induced anti-E75 CD8⁺T cells better recognized murine tumor cells expressing low Her2/neu levels. These results could be explained by the interference of Her2/neu with T cell recognition via an inhibition of HLA class I molecules mediated by an activation of the MAPK pathway (40, 41). An inverse correlation between Her2/neu and MHC class I antigen-processing machinery (APM) component levels in human mammary carcinoma lesions has also been reported supporting this hypothesis (41). Interestingly patients who benefited most from vaccination with the E75 peptide included those who did not express high levels of Her2/neu (3+), but rather low or moderate levels (1+, 2+)(23). Beyond the Her2/neu cancer vaccine, high Her2/neu-expressing tumors from breast cancer patients will be associated with
impaired antigen presentation leading to a poor recognition of tumor antigen by CD8+ T cells and this group of patients is unlikely to respond to cancer vaccines and immunotherapy (40).

In line with these results, we observed that the STxB-E75 vaccine was efficient in a therapeutic setting to inhibit the growth of low-Her2/neu expressing HLA-A2-B16 tumors in HLA-A2 mice, while it showed no significant effect in the control of high-Her2/neu-expressing HLA-A2-B16 tumors both in term of tumor growth and survival analysis. Thus unexpectedly, low expressing Her2 tumors are more responsive to the vaccine than high Her2/neu expressing tumors.

When the STxB-E75 vaccine was combined with anti-Her2/neu mAb, a clear synergy was observed on the control of growth of tumors expressing low or high levels of Her2/neu. However, an impact on survival was mainly observed with the low-Her2/neu expressing tumor model. The lack of effect with the anti-Her2/neu mAb when used alone could be explained by the fact that in this model, Her2/neu is not a natural driver of the tumor, which expresses this antigen only after transfection.

In human, combination of trastuzumab with Her2/neu based vaccines showed a synergistic effect in the induction of potent T cell responses and encouraging clinical results were observed (8). Two phase II clinical trials are ongoing based on these preliminary results (NCT02297698)(NCT01570036). A mechanism that could account for this synergy is the ability of the anti-Her2/neu antibody to induce Her2/neu internalization and degradation, leading to the generation of Her2/neu derived peptides presented by MHC class I molecules to correspondingly specific lymphocytes (42). Indeed, trastuzumab-pretreated breast cancer cells were lysed more efficiently by E75 primed CTL, when compared with untreated breast cancer cells (43). In the current study, we did not observe increased levels of anti-E75 CD8+ T cells when the STxB-E75 vaccine was combined with anti-Her2/neu mAb (data not shown).
A possible limitation of the present study resides in the fact that hHer2/neu is not a self-antigen in humanized HLA-A2 TG mice. To the best of our knowledge HLA-A2-hHer2/neu TG mice are not available. However, we have already demonstrated the ability of STxB coupled to murine Her2/neu derived peptide to break tolerance against Her2/neu as self antigen (44). In addition, spontaneous Her2/neu-specific T-cell responses and antibody responses have been documented in patients with Her2/neu+ tumors (3), and patients treated with peptide-based vaccines have mounted HER2/neu-specific immune responses (23), implying that overcoming immunological tolerance against Her2/neu in patients with breast cancer is feasible.

It might also be argued that the use of a single CD8 peptide exerts less immune pressure than poly-epitopic vaccines, and that the absence of CD4 associated peptides as in the case of STxB-E75, usually does not favor the generation of persistent CD8^+T cells. However, encouraging clinical responses have already been obtained with the E75 peptide alone, as well as with other CD8 peptide-based vaccine strategies (45). Although the use of long peptides incorporating CD4 and CD8 epitopes is often privileged to achieve significant CD8^+T cell responses (17, 46), this study and previously published results from our group clearly document that the STxB delivery vector coupled to various CD8 peptides induces functional and persistent CD8^+T cell responses, thereby bypassing the requirement of CD4 helper signal (44). In addition, epitope spreading has been reported after Her2/neu vaccines administration and the secondary recruitment of a broad anti-tumor T cell repertoire may participate in the clinical response of the mono-epitopic vaccines (8, 47, 48).

Cardiac toxicity has initially represented a clinical concern for the targeting of Her2/neu by mAb and a serious adverse event was reported following the administration of chimeric antigen receptor T cell recognizing Her2/neu in a patient with colorectal cancer (49).
However, all Her2/neu vaccines that were tested in humans alone or in combination with trastuzumab were devoid of significant clinical toxicity.

Regarding the toxicity of STxB, in vitro STxB did not exhibit cytotoxic effect on various normal cell lines and after repetitive administration of high dose of STxB, no toxicity was observed in mice (50).

The analysis of the dynamics of spontaneous or vaccine-induced anti-tumor CD8+T cells clearly showed that in the course of tumor progression, they are anergized via the upregulation of checkpoint inhibitors (PD-1, CTLA-4) or blocked by various immunosuppressive mechanisms including regulatory T cells (51). Reversal of these blocking factors improved the efficacy of a Her2/neu cancer vaccine by reactivating intratumoral T cells leading to improved clinical results (52).

Since from a clinical perspective, Her2/neu cancer vaccines would be combined with anti-Her2/neu mAb, especially breast cancers expressing high levels of Her2/neu, it is important to notice that T cells and immune signatures are crucial for the efficacy of anti-Her2/neu mAb treatments (13, 53). Patients with low intratumoral immune infiltrates will thus likely benefit from a combination of Her2/neu mAb with Her2/neu cancer vaccine to favor T cell infiltration and ideally combined with a blockade of PD-1/PD-L1 pathway (54). In addition, in low Her2/neu expressing tumors, cancer vaccines alone may overcome the clinical resistance to anti-Her2/neu mAb likely because T cells could be activated by a low amount of the HLA-peptide complex, while high levels of Her2/neu are required for the efficacy of antibodies.

**Conclusion**: The body of experiments presented in this work clearly demonstrates that the STxB-E75 vaccine is a potent candidate to be tested in the clinics especially in patients with tumors expressing low levels of Her2/neu. In association with an anti-Her2/neu mAb, the
Her2/neu cancer vaccine may be particularly useful in patients with low intratumoral immune infiltrates, either alone, or in combination with blockers of checkpoint inhibitors.

References


**Figure and Legends**

**Fig 1:** STxB-E75 is more efficient than the E75 peptide to elicit functional anti-Her2\textsubscript{369-377} specific CD8\textsuperscript{+}T cells in HLA-A2 TG mice

HLA-A2 TG mice were immunized at D0 and D14 by the subcutaneous route with STxB-E75 (0,5 nmol) or E75 peptide (Her2\textsubscript{369-377})(25 nmol or 0,5 nmol) or E75 with the flanking sequence (RRAR) present in the STxB vaccine. The vaccines were mixed with GM-CSF at D0 and CpG was injected at day 1.Seven days after the boost, spleen was collected and HLA-A2-Her2\textsubscript{369-377} specific CD8\textsuperscript{+}T cells were detected by dextramer assay (A and B) and IFN\gamma Elispot assay (C).

A) Dextramer analysis of HLA-A2 restricted anti-Her2\textsubscript{369-377} CD8\textsuperscript{+}T cells induced by the various vaccines. Results are represented as the mean ± SD of 4 mice/group from 5 independent experiments

B) Representative dot plots of spleen HLA-A2-Her2\textsubscript{369-377} specific CD8\textsuperscript{+}T cells detected by cytometry. A dextramer control was included in each experiment.
C) Number of IFNγ spot forming cells per 10⁵ CD8⁺T cells detected by Elispot. Background obtained with unpulsed cells was subtracted (number of spots was always<10).

Mean ± SD of 4 mice/group from 3 independent experiments. * P < 0.05, ** P<0.01, *** p < 0.005 ****P<0.0001

**Fig 2: Anti-Her2₃₆₉₋₃₇₇ specific CD8⁺T cells induced by STxB-E75 are multifunctional, of high avidity and persistent**

Specific CD8⁺ splenic T cells were sorted by magnetic beads from HLA-A2 TG mice vaccinated with STxB-E75 (0.5nmol) (in black) or E75 (25nmol) (in grey), then specific HLA-A2/Her2₃₆₉₋₃₇₇ CD8⁺T cells were sorted by flow cytometry after dextramer staining.

A) HLA-A2 restricted Her2₃₆₉₋₃₇₇ CD8⁺T cells (5000 cells/well) were cocultured with antigen presenting cells (APC) (CD3 negative cells) pulsed with medium (dashed histogram) or Her2₃₆₉₋₃₇₇ peptide (10 μg/ml) (filled histogram). Cytokines and chemokines were measured by Luminex assay performed on the supernatant after 18h. Mean ± SD of 3 mice/group were represented. This experiment is representative of two experiments. *P<0.05

B) Splenocytes from HLA-A2 TG mice were labeled with 5 μM CFSE (CFSE<sup>high</sup>) and pulsed with the E75 peptide at 10 μg/ml or labeled with 1μM CFSE (CFSE<sup>low</sup>) and used as non-pulsed target cells. CFSE-labeled cells containing an equal number of cells from each fraction (CFSE high and low) were injected i.v into mice previously immunized with E75 (25 nmol) or STxB-E75 (0.5 nmol). After 15-18h, single-cell suspensions from spleens were analyzed by flow cytometry. The percentage of specific killing was determined according to the following calculation: [1 - ((CFSE<sup>low</sup> / CFSE<sup>high</sup> of naive mice) / (CFSE<sup>low</sup> / CFSE<sup>high</sup> of vaccinated mice))] x100. Data are shown as a representative dot plot from each group (top row) or as a bar graph displaying the means ± SD from 9 mice/group. *P<0.01
C) HLA-A2 restricted Her2\textsubscript{369-377} CD8\textsuperscript{T} cells (1500 cells/well) were stimulated in the presence of APC with decreasing concentrations of Her2\textsubscript{369-377} peptide and the number of IFN\textgamma producing cells was revealed by Elispot. Mean ± SD of 3 mice/group were represented. This experiment is representative of two experiments. *P < 0.05

D) Mice were vaccinated at D0 and 14 with STxB-E75 (0.5 nmol) or E75 peptide (25 nmol). The first vaccine dose was mixed with GM-CSF at D0 and CpG was injected at day 1. Thirty days after the boost, HLA-A2/Her2\textsubscript{369-377} specific CD8\textsuperscript{T} cells were detected by dextramer assay (left) and IFN\textgamma Elispot assay (right) as in figure 1. Means ± SD of 12 mice/group from 3 independent experiments. *** P<0.001

**Fig 3**: STxB-E75 inhibits the growth of B16-HLA-A2/Her2 tumor in a therapeutic and prophylactic setting via a CD8 dependent mechanism.

Mice were grafted with B16-HLA-A2-HER2/neu (1.5x10\textsuperscript{6} cells) and then treated or not with STxB-E75 (0.5 nmol) or E75 (0.5 nmol) by the s.c route at day 3 and day 10. The first vaccine dose was combined with GM-CSF (20 \textmu g) and CpG (50 \textmu g) was administered at day 4. The growth (A) and survival (B) of mice were monitored.

Mice (n = 6) were immunized or not by the s.c route with STxB-E75 (0.5 nmol) or E75 (0.5 nmol) at day 0 and 14 combined with GM-CSF (20 \textmu g) at day 0 and CpG (50 \textmu g) at day 1. At day 21, mice were grafted or not with B16-HLA-A2-HER2 (1.5.10\textsuperscript{6} cells) and the growth (C) and survival (D) of mice were monitored.

Mice were grafted with B16-HLA-A2-HER2/neu (1.5x10\textsuperscript{6} cells) and then treated or not with STxB-E75 (0.5 nmol) by the s.c route at day 3 and 10 combined with GM-CSF (20 \textmu g) at day 3 and CpG (50 \textmu g) at day 4. For in vivo depletion, anti-mCD8 mAb (100 \textmu g) was
injected i.p once a week, beginning 2 days before the first vaccination. The growth (E) and survival (F) of mice were monitored. All of these experiments have been reproduced at least two times and the growth and survival of mice were monitored twice per week. Each group included 6 mice. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig 4: Anti-Her2/neu CD8+ T cells preferentially recognize low Her2/neu-expressing tumor cell lines.

CD8+ splenic T cells were isolated from HLA-A2 TG mice vaccinated with STxB-E75 using magnetic beads and HLA-A2 restricted Her2369-377 CD8+ T cells were sorted by flow cytometry after dextramer staining. IFNγ Elispot assay was performed after co-culturing for 24 hours HLA-A2 restricted Her2369-377 CD8+ T cells (5000 cells/well) with B16-HLA-A2 expressing low or high Her2/neu (A) or with human tumor cell lines expressing variable levels of Her2/neu previously treated or not with IFNγ (100 IU) for 48 hours (B). Data are expressed as means of triplicates ±SD. Experiments are representative of three experiments. Cells treated with IFNγ were washed before being transferred in the wells.

Fig 5: Anti-Her2/neu CD8+ T cells preferentially recognize low Her2/neu-expressing tumor cell lines.

Mice were grafted with B16-HLA-A2-Her2/neuLow (1.5x10^6 cells) (A and B) or B16-HLA-A2-Her2/neuHigh (C and D) and then treated or not with STxB-E75 (0.5 nmol, s.c) and/or anti-Her2 mAb (4D5) (100 μg, i.p) at day 3 and 10. The first vaccine dose was combined with GM-CSF (20 μg)(day 3) and CpG (50 μg) was administered at day 4.
The growth (A and C) and survival (B and D) of mice were monitored. Each group included 6 mice and results shown are representative of two experiments. * P < 0.05, ** P < 0.01, **** P < 0.0001.
Figure 2

A

B

Vaccine

Pulse

STxB-E75

E75

medium

E75 peptide

medium

E75 peptide

Naive mice

STxB-E75

E75

% specific lysis

0 10 20 30 40 50 60 70 80

IL-2 IL-4 IL-10 IL-12 (p40) IL-12 (p70) IL-17 KC RANTES IL-13 IL1β TNFα MIP1α MIP1β

Pg/ml

0 20 40 60

IL-2 IL-4 IL-10 IL-12 (p40) IL-12 (p70) IL-17 KC RANTES IL-13 IL1β TNFα MIP1α MIP1β

0 50K 100K 150K 200K 250K

CFSE

0 10^3 10^4 10^5 10^6

Naive mice

STxB-E75

E75

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Figure 2

C

![Graph showing the number of IFN-γ producing cells/1500 HLA-A2-Her2369-377 specific CD8 cells against various concentrations of Her2369-377 (μg/ml).](image)

D

![Graph showing the percentage of HLA-A2/Her2369-377 tetramer/CD8 T cells and the number of IFN-γ producing cells/10^5 CD8 T cells for STXB-E75 and E75 at different concentrations.](image)

**Research.**

[Note: The figures are not fully visible in the image, but they likely depict experimental data related to immune responses and cell counts.]
Figure 3

(A) Tumor size (mm²) over time for different vaccine combinations:
- PBS
- E75
- StxB-E75

(B) Survival rates over time:
- PBS
- StxB-E75
- E75

(C) Tumor size (mm²) over time for different vaccine combinations:
- PBS
- E75
- StxB-E75

(D) Percent survival over time:
- PBS
- E75
- StxB-E75

Research.

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Figure 3
Figure 5
A therapeutic Her2/neu vaccine targeting dendritic cells preferentially inhibits the growth of low Her2/neu-expressing tumor in HLA-A2 transgenic mice

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