Supplemental materials and methods

Mice. Female BALB/c^{wt} (H-2^{d})**,** C57BL/6^{wt} (H-2^{b}), CB6F1^{wt} (H-2$^{d/b}$), transgenic BALB-neuT and Nod SCID mice were from Charles River Laboratory (Calco, Italy) while FVB^{Her2} from Genentech (San Francisco, CA). CB6F1^{neu} mice transgenic for the *neu* gene were generated by us by crossing BALB-neuT males (1) with C57BL/6^{wt} females. CB6F1^{Her2} mice were generated by crossing C57BL/6^{Her2} males expressing *Her-2* gene under the whey acidic protein promoter (2) with BALB/c^{wt} females. Genotyped and individually tagged female mice of the same age were treated according to the EU guidelines.

Confocal microscopy. 3T3 fibroblasts transiently tranfected with HuHuT and RRT were cultured in DMEM at 10% fetal bovine serum (FBS, Invitrogen, Life Technologies, Carlsbad, California) for 24 hours. Then, cells were plated on glass coverslips and grown to confluency. Coverslips were then washed and incubated for 1 hour at 37°C with sera (diluted 1:20) from immunized mice. Cells were then fixed for 5 minutes with PBS–4% paraformaldehyde (Sigma-Aldrich, Milan, Italy), permeabilized for 7 minutes with PBS–0.2% Triton X-100 (Sigma-Aldrich), and blocked with PBS–10% BSA (Sigma-Aldrich) for 20 minutes. Membrane and cytoplasmic expression of RRT and HuHuT proteins was assessed by staining with Alexa Fluor 488–conjugated goat anti-mouse IgG (1 hour, 2 μg/ml; Molecular Probes Inc., Eugene, Oregon, USA). After rinsing thrice with PBS, the coverslips were air dried and mounted with Mowiol 4-88 (Calbiochem). Samples were visualised with a confocal laser scanning microscopy system equipped with an argon-ion laser (LSM510; Zeiss). Photographs were taken using a CCD-300-RC camera and images were processed using Adobe Photoshop and Microsoft PowerPoint software.

RNA isolation and reverse transcriptase (RT)-PCR amplification. Total RNA was extracted and purified from thymus of CB6F1^{wt}, CB6F1^{neu} and CB6F1^{Her2} mice using TRIZOL reagent (Invitrogen). cDNA was synthesized with Supercript first strand cDNA System (Invitrogen) following the manufacturer's instructions. PCR for *neu* was performed by using the following specific primers: 5'-ATTCATCATTGCAACTGTAGA-3' and 5'-AAGCACCTTCACCCTTCCTTA-3'. For Her-2 different primers were used: 5'-GTCCATCATCTCTGCGGTGGT-3' and 5'-AAGCACCTTCACCCTTCCTCA-3'. Primers that amplify beta actin (BD Clontech) were used as standard.

Whole mounts. At week 10, 14 and 18 five mice from each group were sacrificed and their mammary tissue morphologically analyzed. Mice pelts were fixed for 4 h in periodate-lysine paraformaldehyde

(PLP), washed in PBS containing 20% of sucrose, processed for whole mount preparations (3). Pictures were taken with a Nikon Coolpix 950 digital camera (Nital S.p.A., Turin, Italy) mounted on a stereoscopic microscope (MZ6; Leica, Milan, Italy) with a 0.63 objective giving a total magnification of ×6.3. For each time point, 1 out of 5 independent images is represented.

Immunization and evaluation of tumor growth. Anesthetized mice were vaccinated as previously described (3). The vaccination course consisted of two i.m. injections of 50 μg of plasmid, followed by electroporation with an interval of 14 days. Vaccination course were started at week 10 in CB6F1^{neu} mice and at week 20 in FVB^{Her2} mice and it was repeated each ten weeks. The mammary pad of immunized mice was inspected weekly by palpation to note tumor appearance. Tumor masses were then measured with a cailiper. Progressively growing masses >1 mm mean diameter were regarded as tumors. Mice were sacrificed when one of the tumors exceeded 10 mm diameter or when mice presented ten tumors. Tumor incidence is expressed as the percentage of mice free of palpable tumors in function of time. Tumor multiplicity was calculated as the cumulative number of incident tumors/total number of mice for each week; values are reported as mean ± SE. Overall survival is expressed as the percentage of alive mice in function of time. Differences in tumor incidence were analyzed by the Log-rank (Mantel-Cox) test and Fisher's exact test.

Cell lines. 3T3/KB, 3T3/NKB or 3T3/EKB cells were obtained by transfecting BALB/c 3T3 fibroblasts with H-2K^d and B7.1. Moreover 3T3/NKB and 3T3/EKB cells were also transfected with neu or Her-2 respectively (4). These cell lines were cultured in Dulbecco's modified Eagle's medium with Glutamax 1 (DMEM, Life Technologies, San Giuliano, Italy) supplemented with 20% heat-inactivated FBS. 3T3/NKB and 3T3/EKB cells were cultured with 0.6 mg/ml G418 (Geneticin, Invitrogen) and 0.6 mg/ml zeocin (Invitrogen), whereas 3T3/KB cells with 0.6 mg/ml G418 and 7.5 μg/ml puromycin (Invitrogen). TUBO^{neu} carcinoma cells expressing H-2 K^d and neu molecules are from a mammary carcinoma arisen in a BALB/c female mouse transgenic for the activated *neu* (BALB-neuT mice) (5). SKOV3 cells, a human ovary cancer cell line overexpressing Her-2, were from the ATCC. These cells were cultured at 37°C and 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium with Glutamax 1 supplemented with 20% heat-inactivated FBS.

IFN-γ ELISPOT assay. Two weeks after vaccination, SPC were added to the wells of 96-well HTS IP plates (Millipore, Bedford, MA) pre-coated with 5 μg/ml rat anti-mouse IFN-γ (clone R4-6A2, BD Biosciences, San Jose, CA). SPC were incubated for 48 hours with 3T3/KB, 3T3/NKB or 3T3/EKB at an

APC:lymphocyte ratio of 1:10. IFN-γ spots were enumerated as previously described (6). Data were analyzed by the Student's *t* test.

Antibody response. Sera collected two weeks after the second vaccination were diluted 1:200 in PBS and 100 μl were incubated for 30 minutes at 4°C with SKOV3 cells for 15 minutes at 4°C. Total Ig binding was evaluated using a FITC-conjugated goat anti-mouse IgG Fc secondary antibody (DakoCytomation). The Ab5 (Calbiochem, San Diego, CA) mAb were used as positive controls for neu and Her-2 positivity respectively. Flow cytometry was performed with a CyAn ADP (DakoCytomation). The results were expressed as mean fluorescence intensity (MFI) and analyzed with Summit 4.2 (DakoCytomation) software. Differences in MFI were analyzed by Student's *t* test.

Winn-type assay. To determine the role of $CD4^+$ and $CD8^+$ T cells in the spleen from vaccinated animals, $CD4^+$ and $CD8^+$ T cells with were first isolated from the spleens of $CB6F1^{wt}$ immunized mice using the $CD4^+$ and $CD8^+$ T Cell isolation kit (Miltenyi Biotec, Auburn, CA). The resultant cell population contained >90% $CD4^+$ and >85% $CD8^+$ cells by flow cytometry. These $CD4^+$ and $CD8^+$ T lymphocytes were mixed with viable $TUBO^{neu}$ tumor cells at a 1:10 tumor:lymphocyte ratio. The mixture (containing 1×10^5 tumor cells, 1×10^6 $CD4^+$ or $CD8^+$ T cells) was added with $2x10^4$ IU of mouse recombinant IL2 (PeproTech Inc., Rocky Hill, NJ, USA) and inoculated into the flanks of NOD SCID or $CB6F1^{wt}$ naïve mice. At day +1, +2 and +3 from tumor challenge $2x10^4$ IU of mouse recombinant IL2 (PeproTech Inc.) was injected i.p. into Nod Scid mice. Mice were observed on a daily basis, and tumor growth was measured with a caliper; n= 3-7 mice per group.

Immunohistochemistry. 24 days after tumor challenge three mice from each experimental group were sacrificed and their mammary fat pad in which the challenge was performed was fixed for 2 h in PLP, washed in PBS containing 20% of sucrose and processed for immunoistochemistry evaluation. Six –micron cryostat section were air- dried and fixed in ice-cold acetone for 10 min. The anti-CD4, anti-CD8 primary antibodies used for staining were all from Abcam - Cambridge, UK. Slides were then incubated with the appropriate biotinylated secondary antibody : goat anti –rat IgG (Jackson ImmunoResarch Laboratories, Inc., West Grove, PA) at 1:500 dilution for 30 minutes. Immunoreactive antigens were detected using NeutrAvidin™ Alkaline Phosphatase Conjugated (Thermo Scientific-Pierce Biotechnology, Rockford, USA) and Vulcan Fast Red (Biocare Medical, Concord, CA).

Molecular modeling. All the calculations have been carried out on Silicon Graphics O2 R12000 workstations, using the InsightII (Accelrys, Inc.) software package. RHuT and HuRT protein structures

was constructed with homology modeling using the extracellular portion of neu and Her-2 (1n8yc and 1n8zc) as template and Swiss-Model Server with SPDBV program suite. The structures was then submitted to minimization cycles, using a Newton-Raphson minimization algorithm with at least 5000 iterations. The minimization cycles were repeated until energy global minimum reached. The models obtained were checked using standard validation programs as PROCHECK e VERIFY_3D (7-9). The prediction of conformational epitopes has been carried out on the proteins RHuT , HuRT, HuHuT (1n8z) and RRT (1n8y). Conformational epitopes prediction has been calculated using BEpro (PEPITO) that take advantage from amino-acid propensity scores and of half sphere exposure values at multiple distances (10). The folding was evaluated measuring euclidean distance between homologous atoms, precisely between α-carbon using a bioinformatic tool that was developed by prof. Mauro Angeletti at University of Camerino.

Supplemental figures legends

Figure S1: Sera from mice immunized with RHuT and HuRT better down-regulate membrane RRT and HuHuT protein, respectively. 3T3 fibroblasts transfected with RRT and HuHuT were incubated with the immune sera from CB6F1^{wt} mice vaccinated with RRT and RHuT (left) while 3T3 fibroblasts transfected with HuHuT were incubated with the immune sera from mice vaccinated with HuHuT and HuRT (right). Cytoplasmic dots stained with Alexa Fluor 488–conjugated goat anti-mouse IgG show protein internalization. A representative experiment from three experiments performed independently. Magnification: x63.

Figure S2: neu protein expression and progression of *neu*-dependet mammary carcinogenesis in CB6F1^{neu} mice. *A*, neu and Her-2 mRNA expression in the thymus of 4-week-old CB6F1^{wt}, CB6F1^{neu} and CB6F1$^{Her\text{-}2}$ mice. Neu expression was detected in thymus samples of CB6F1^{neu} mice and in TUBO^{neu} cells, whereas no signal was observed in the thymus of CB6F1^{wt} mice and in neu negative TSA and D2F2/E2$^{Her\text{-}2}$ cells. By contrast, Her-2 expression was evident in the thymus of CB6F1^{Her2} mice and in D2F2/E2^{Her2} cells whereas it was not evident in the thymus of CB6F1^{wt} mice and in Her-2 negative TSA and TUBO^{neu} cells. *B*, authocthonous mammary carcinogenesis in CB6F1^{neu} mice is shown as tumor incidence (*left*), tumor multiplicity (*middle*) and overall survival (*right*). *C*, whole-mount images of representative 10, 14 and 18-week-old CB6F1^{neu} mammary glands. Epithelial nodular side buds stemming from the main and secondary mammary ducts are evident at 10 weeks of age (*left*). At

week 14, these foci of atypical hyperplasia have grown to lesions similar to *in situ* carcinomas (*middle*). Progressively these lesions speed over the whole of the mammary gland and converge to form invasive carcinomas that become palpable around week 18 (*right*).

Supplementary figure S3. Life-long antitumor protection afforded by repeated vaccination in CB6F1^{neu} mice. Mice were vaccinated every 10 weeks throughout their life, starting when their mammary glands displayed atypical hyperplasia (week 10). They were boosted with empty pVAX plasmid (dotted grey line; 8 mice) or with RHuT plasmid (○; 9 mice).

Supplementary figure S4: Cellular response against neu (*left*) and Her-2 (*right*) displayed by CB6F1^{neu} mice. Two weeks after vaccination with RHuT, HuRT, RRT and HuHuT the number of IFN-γ producing after in vitro restimulation with 3T3/KB (grey bars), 3T3/NKB (black bars) and 3T3/EKB (white bars) cells was evaluated by Elispot assay and expressed as spot forming unit (SFU).

Supplementary figure S5: Immune response against Her-2 elicited in FVB^{Her2} mice by RHuT and HuRT vaccination. *A:* protection against autochthonous Her-2$^+$ mammary tumors provided by RHuT (○, 5 mice) and HuRT (□, 7 mice) electroporation started at 20 weeks of age. Incidence of mammary tumor was followed in untreated mice (dotted grey line, 10 mice) as control. Tumor incidence in RHuT and HuRT vaccinated mice is significantly different as compared with untreated mice (p = 0.08 and p=0.05 respectively, Log-rank Mantel-Cox test). *B:* anti-Her-2 antibodies in the sera of the same mice. Results are represented as MFI $\pm$ SE; *, $p < 0.0001$ by Student's t test.

Supplementary figure S6: Antitumor reactivity of lymphocyte subpopulations from CB6F1^{wt} mice immunized with RRT and RHuT evaluated in a Winn-type assay. Nod SCID (*A and B*) and CB6F1^{wt} (*C and D*) mice were injected subcutaneously in the fat pad area with 1 x 10^5 TUBO^{neu} cells admixed with 1x10^6 CD8$^+$ (*A*) or CD4$^+$ T (*B, C and D*) cells from control pVAX (dotted grey lines), RRT (black circles) and RHuT (empty circles). *B*: tumor incidence in the presence of CD4$^+$ T cells from RRT and RHuT vaccinated mice is significantly different from that observed in the presence of CD4$^+$ cells from control pVAX immunized mice ($p < 0.0001$, Log-rank Mantel-Cox test). *C* and *D*: At day 24 the number of tumor-free CB6F1^{wt} mice receiving TUBO^{neu} cells admixed with CD4$^+$ T cells from RHuT vaccinated

mice is significantly different from that of mice receiving TUBO^{neu} cells admixed with CD4^{+} T cells from RRT vaccinate mice (p=0.03, Chi-square test).

Supplementary figure S7: Histological features and immunohistochemistry of the growth and rejection pattern of TUBO^{neu} tumors admixed with CD4^{+} lymphoid cells from CB6F1^{wt} mice vaccinated with control pVAX (*A*-*C*), RRT (*D*-*F*) and RHuT plasmids (*G-I),* evaluated 24 days after challenge. Hematoxilin and eosin (H&E) staining shows more frequent and larger that necrotic areas when TUBO^{neu} cells are admixed with CD4^{+} cells from RHuT vaccinated mice (*G*) as compared to what is observed when tumor cells are admixed with CD4^{+} cells from mice immunized with pVAX (*A*) and RRT (*D*). The marked CD4^{+} (*E and H*) and CD8^{+} (*F and I*) reactive infiltrate present when TUBO^{neu} cells were admixed with CD4^{+} cells from RRT and RHuT immunized mice are similar. A less evident infiltrate is evident when TUBO^{neu} cells are admixed with CD4^{+} cells from control pVAX immunized mice.

Supplementary figure S8: Molecular models of RRT, RHuT, HuRT and HuHuT proteins and epitope prediction. Candidate regions to act as conformational epitopes are shown in red.

References

1. Boggio K, Nicoletti G, Di Carlo E*, et al.* Interleukin 12-mediated prevention of spontaneous mammary adenocarcinomas in two lines of Her-2/neu transgenic mice. J Exp Med 1998;188(3):589-96.

2. Piechocki MP, Ho YS, Pilon S, Wei WZ. Human ErbB-2 (Her-2) transgenic mice: a model system for testing Her-2 based vaccines. J Immunol 2003;171(11):5787-94.

3. Quaglino E, Iezzi M, Mastini C, *et al.* Electroporated DNA vaccine clears away multifocal mammary carcinomas in Her-2/neu transgenic mice. Cancer Res 2004**;**64(8):2858-64.

4. Jacob J, Radkevich O, Forni G*, et al.* Activity of DNA vaccines encoding self or heterologous Her-2/neu in Her-2 or neu transgenic mice. Cell Immunol 2006;240(2):96-106.

5. Rovero S, Boggio K, Carlo ED*, et al.* Insertion of the DNA for the 163-171 peptide of IL1beta enables a DNA vaccine encoding p185(neu) to inhibit mammary carcinogenesis in Her-2/neu transgenic BALB/c mice. Gene Ther 2001;8(6):447-52.

6. Mastini C, Becker PD, Iezzi M*, et al.* Intramammary application of non-methylated-CpG oligodeoxynucleotides (CpG) inhibits both local and systemic mammary carcinogenesis in female BALB/c Her-2/neu transgenic mice. Curr Cancer Drug Targets 2008;8(3):230-42.

7. Luthy R, Bowie JU, Eisenberg D. Assessment of protein models with three-dimensional profiles. Nature 1992;356(6364):83-5.

8. Morris AL, MacArthur MW, Hutchinson EG et al. Stereochemical quality of protein structure coordinates. Proteins 1992;12(4):345-64.

9. Laskowski RA, Rullmannn JA, MacArthur MW et al. AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. J Biomol NMR 1996;8(4):477-86.

10. Sweredoski MJ, BaldiP. PEPITO: improved discontinuous B-cell epitope prediction using multiple distance thresholds and half sphere exposure. Bioinformatics 2008;24(12):1459-60.