Gene-specific inhibition of breast carcinoma in BALB-*neu*T mice by active immunization with rat Neu or human ErbB receptors

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Abstract. Employing the transgenic BALB-*neu*T mouse tumor model, we explored the *in vivo* biologic relevance of immunocompetent epitopes shared among the four ErbB receptors. The outcome of *neu*-mediated tumorigenesis was compared following vaccination with isogeneic normal rat ErbB2/Neu (LTR-Neu) or xenogeneic human ErbB receptors (LTR-EGFR, LTR-ErbB2, LTR-ErbB3 and LTR-ErbB4), each recombinantly expressed in an NIH3T3 murine cell background. Vaccination using rat LTR-Neu at the stage of atypical hyperplasia potently inhibited neu-mediated mammary tumorigenesis. Moreover, all human ErbB receptors specifically interfered with tumor development in BALB-*neu*T mice. Relative increase in tumor-free survival and reduction in tumor incidence corresponded to structural similarity shared with the etiologic *neu* oncogene, as rat orthologue LTR-Neu proved most effective followed by the human homologue LTR-ErbB2 and the other three human ErbB receptors. Vaccination resulted in high titer specific serum antibodies, whose tumor-inhibitory effect correlated with cross-reactivity to purified rat Neu extracellular domain *in vitro*. Furthermore, a T cell response specific for peptide epitopes of rat Neu was elicited in spleen cells of mice immunized with LTR-Neu and was remotely detectable for discrete peptides upon vaccination with LTR-ErbB2 and LTR-EGFR. The most pronounced tumor inhibition by LTR-Neu vaccination was associated with leukocyte infiltrate and tumor necrosis *in vivo*, while immune sera specifically induced cytotoxicity and apoptosis of BALB-*neu*T tumor cells *in vitro*. Our findings indicated that targeted inhibition of *neu* oncogene-mediated mammary carcinogenesis is conditional upon the immunization schedule and discrete immunogenic epitopes shared to a variable extent by different ErbB receptors.

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Introduction

Members of the epidermal growth factor (EGF) receptor family of tyrosine kinases that includes EGFR, ErbB2, ErbB3 and ErbB4 have been implicated as specific molecular targets in human neoplasia by overexpression in the presence or absence of gene amplification (1). High-level overexpression of ErbB2 or EGFR in carcinoma as compared to normal tissue has proven both etiological for tumorigenesis and amenable to targeted intervention in patients with breast and other common epithelial malignancies, using monoclonal antibodies or small molecule inhibitors *in vivo* (1,2). In addition, accumulating evidence indicates development of a spontaneous immune response in some patients with tumors harboring ErbB receptor overexpression (3-5). In various human malignancies of epithelial origin including breast carcinoma, an immune response to ErbB2 affected multiple combinations of ErbB receptors simultaneously (6). Extensive sequence conservation among the ErbB receptor family on one hand or high propensity of functional interaction by heterodimerization on the other hand have invoked alternative possibilities of either common immunogenic epitopes conserved to a variable extent among different ErbB receptors

or genuine individual immune responses to different ErbB receptors prompted by functional cooperation in the neoplastic process *in vivo*.

BALB-*neu*T mice that are transgenic for the *neu* oncogene, represent a suitable animal model to study cross-vaccination efficiency of different ErbB receptors as they exhibit reproducible transition from normal epithelium to multifocal breast carcinoma in a constrained temporal order and at 100% efficiency of tumor formation caused by the activated rat homologue of ErbB2 (7). In relation to the etiologic mature rat Neu product (8), its human orthologue ErbB2 (9) shares 87% overall identity or 92% similarity in primary protein structure. By comparison, human EGFR (10) exhibits 49% identity or 63% similarity, ErbB3 (11) 37% identity or 50% similarity and ErbB4 (12) 43% identity or 57% similarity. The degree of structural conservation within the ErbB family becomes even more evident when comparing collinear homology from amino terminus throughout the carboxyl terminal border of the tyrosine kinase domain, thus excluding the carboxyl terminal signal transfer domain representing the region of highest divergence. In this case, the respective extent of identity or similarity with rat Neu is 89% or 93% for ErbB2, 54% or 68% for EGFR, 46% or 61% for ErbB3 and 51% or 66% for ErbB4. Comparing extracellular domains of ErbB receptors, which due to cell surface exposure are readily accessible *in vivo* by the host's immune system, identity or similarity of human ErbB receptors with rat Neu are 85% or 91% for ErbB2, 42% or 57% for EGFR, 41% or 55% for ErbB3 and 40% or 56% for ErbB4, respectively. Tumor vaccine studies have utilized the BALB-*neu*T model in the past, which included different delivery strategies employing DNA, synthetic peptides, purified recombinant protein, adenoviral vector or a cell-based vaccine. Targeting rat Neu-specific sequences these approaches have been shown to protect BALB-*neu*T mice to a variable degree from *neu* oncogene-induced tumorigenesis (13-17).

In the current study we utilized NIH3T3 mouse fibroblasts as vaccination vehicle to deliver high recombinant protein levels of normal rat Neu in LTR-Neu (18) or the four human ErbB receptors including LTR-EGFR (19), LTR-ErbB2 (20), LTR-ErbB3 (21) and LTR-ErbB4 (22). Since biochemical and biological properties of recombinant ErbB receptors have previously been characterized in these cell sources, we could warrant a comparably high antigen dosage as well as structural and functional integrity of distinct ErbB receptors. At similar vaccination efficiencies of different ErbB receptors we demonstrated specific inhibition of *neu* oncogene-mediated mammary carcinogenesis in BALB-*neu*T mice. The extent of interference with tumorigenesis concurred with relative structural conservation of individual ErbB receptors with the tumor-causing *neu* oncogene. Our observations suggested shared biologically relevant, immunogenic epitopes in different ErbB receptors, which can be recruited by cross-vaccination *in vivo*, albeit at substantially reduced efficiency when compared to the autologous receptor protein. Most effective gene-specific protection against tumor development depended on initiation time of vaccination and was accompanied by the development of humoral and cellular immunity resulting in tumor necrosis *in vivo* and induction of apoptosis in BALB-*neu*T tumor cells *in vitro*.

Materials and methods

Antibodies, peptides and cells. Polyclonal anti-ErbB2 peptide antiserum M6 has previously been characterized (20). Polyclonal antisera to EGFR, ErbB3 and ErbB4 receptors from mouse, rat and human origin, were purchased from Santa Cruz Biotechnology (anti-EGFR 1005, anti-ErbB3 C-17, anti-ErbB4 C-18, Santa Cruz Biotechnology, CA, USA). Mouse monoclonal antibodies (Mab) to CD20, CD15, CD4 and CD8 were acquired from Dako Corporation, Carpenteria, CA. The anti-CEA Mab R4 was previously described (23). Synthetic peptides located in the extracellular (ECD) (Neu 15.3, aa 66-74; Neu 42, aa 169-183; Neu 98, aa 393-407; Neu 141, aa 566-580; Neu 156, aa 626-640), transmembrane (TMD) (Neu 166, aa 666-680) or intracellular domains (ICD) (Neu 209, aa 838-852; Neu 231, aa 926-940; Neu 301, aa 1206-1220) of rat Neu sequence (8, NCI, PubMed Accession 1202344A) were provided by IRBM P. Angeletti (Pomezia, Rome). Neu-overexpressing BALB-*neu*T mammary cancer cells (H-2d) (TUBO) were previously described (13). NIH3T3 cells encoding normal rat Neu (LTR-Neu) have been previously characterized and kindly provided by Dr Eddi Di Marco (Istituto Tumori di Genova) (18). Characterization of NIH3T3 parental cells and NIH3T3 cells transfected with expression vectors for human coding sequences of human ErbB family receptors including LTR-EGFR, LTR-ErbB2, LTR-ErbB3 and LTR-ErbB4 has been reported (19-22).

Transgenic BALB-neuT mouse colony. Transgenic BALB-*neu*T male mice (13) were routinely mated with BALB/c females (H-2d; Charles River, Calco, Italy) in the animal facilities of Tor Vergata's University. Progenies were confirmed for presence of the transgene by PCR (13). Individually tagged virgin females were used in this study. Mice were bred under pathogen-free conditions and handled in compliance with European Union and institutional standards for animal research.

Vaccination and analysis of anti-tumor activity in vivo. Groups of BALB-*neu*T mice were vaccinated by i.p. injection of $5x10^6$ NIH3T3, LTR-Neu, LTR-EGFR, LTR-ErbB2, LTR-ErbB3 and LTR-ErbB4 live cells or 500 μl PBS (phosphate-buffered saline) vehicle, starting at the age they display atypical breast hyperplasia (6 weeks). Mice were then boosted three times at four-week intervals (complete immunization schedule: 6, 10, 14, 18 weeks). Other groups of BALB-*neu*T mice were vaccinated with LTR-Neu or NIH3T3 starting at the age coinciding with *in situ* (11 weeks) or invasive breast carcinoma (16 weeks). These mice were boosted three times every four weeks (complete schedule: 11, 15, 19, 23 or 16, 20, 24, 28 weeks). For each immunogen, groups of 8-16 mice were vaccinated. Mammary glands were checked weekly and tumors recorded at 3 mm in diameter. Tumor growth was monitored until all mammary glands displayed a palpable tumor or tumor mass exceeded 15 mm in diameter. At this point mice were sacrificed. Time of initial tumor appearance as well as tumor multiplicity were averaged as the mean ± standard deviation of incident tumors (13).

Immunohistochemical and ultrastructural analysis. Mammary tissue from 2-3 BALB-*neu*T mice at different ages was

processed for immunohistochemical, histological and ultrastructural analyses as previously described (24,25). Three or four tumors were used for each group of vaccinated mice. For immunohistochemistry sections were incubated with the anti-ErbB receptor polyclonal antibodies (1 μg/ml) or with Mabs to different leukocyte markers (CD4, CD8, CD20 and CD15, 1:50 of undiluted supernatants) after antigen retrieval. For mouse antibodies, the reaction was revealed using a mouse to mouse kit (UCS, Morlupo, Italy). Purified immunoglobulins from normal rabbit serum (1 μg/ml) or anti-CEA Mab R4 (1:50 of undiluted supernatant) (23) served as controls. Leucocytes were counted at x400 in 10 microscopic randomly chosen fields. Results represent mean ± SD of positive cells/field evaluated by immunohistochemistry.

Antibody immunity following vaccination with LTR-Neu and LTR-ErbB transfectants. Sera from vaccinated BALB-*neu*T mice were collected prior to immunization and seven days after the final boost. The presence of antibodies reacting with individual ErbB receptor immunogens was assayed using NIH3T3, LTR-Neu, LTR-EGFR, LTR-ErbB2, LTR-ErbB3 or LTR-ErbB4 by immunoblotting or ELISA (enzyme linked immunosorbent assay) as previously described (6,23). For immunoprecipitation, 2 μl of mouse serum and 50 μl of protein G sepharose were incubated with 300 μg lysate of LTR-transfectants or NIH3T3. Lysate (100 μg) of LTR-transfectants was used for Western blotting. For ELISA, mouse serum at different dilutions (1:100, 1:350, 1:1225; 1:4287) was pre-adsorbed five times for 1 h each with $5x10^4$ NIH3T3 cells/well on 96-well plates and then assayed against individual LTR-ErbB transfectants ($5x10^4$ cells/well) and NIH3T3 control. Antibody titers to recombinant ErbB receptor immunogens were determined by ELISA (26).

Antibody titers to purified Neu extracellular domain (Neu-ECD) and immunoglobulin subclasses, were determined by ELISA using a Mouse Typer isotyping kit (BioRad, Richmond, CA, USA). Recombinant ECD-Neu protein was produced in *E. coli* (27) and tested at dilutions of 1:100 to 1:1,000.

Biologic activity of vaccinated mouse immune sera in vitro. Antibody-dependent cellular cytotoxicity (ADCC) was conducted as previously described (17,28). BALB-*neu*T mammary tumor cells ($5x10^3$ cells/well) were used as targets (T), while spleen cells from normal BALB/c mice were used as effectors (E) at 50:1 (E:T ratio). Dilutions (1:15, 1:30, 1:60) of sera pooled from six mice vaccinated with LTR-Neu, LTR-ErbB2 or NIH3T3 were assayed. Percentage of specific lysis was calculated as described (28).

For cell proliferation of BALB-*neu*T cancer cells (29), immunoglobulins from LTR-Neu, LTR-ErbB2 and NIH3T3 pooled sera were purified by protein G and dialyzed against PBS. Purity was determined by SDS-PAGE and Coomassie blue staining. BALB-*neu*T cancer cells ($2.5x10^3$ cells/well) were incubated in serum-free DMEM containing 0.2% BSA containing Ig (10 or 1 μg/ml) or fetal bovine serum (5% or 1%). Ig were replenished every 24 h. All treatments were performed in triplicate. The percent change in relative cell number was calculated as described by Yip *et al* (29)

In situ detection of programmed cell death of BALB-*neu*T cancer cells upon Ig stimulation was determined by a MEBSTAIN apoptosis kit (Immunotech, France). Cells were plated at $5x10^3$ cells/well in chamber slides (Lab-Tek Chamber slide) and incubated with LTR-Neu and NIH3T3 Ig as described above. Staurosporine at 1 μM for 24 h was used as positive control. The percentage of apoptotic cells was calculated by determining the merge positive cells/total cells evaluating 5 randomly chosen microscopic fields.

IFN-γ and IL-2 release assay. Spleen cells from BALB-*neu*T vaccinated mice were harvested 7 days after the final immunization as previously described (26,30). Spleen mononuclear cells ($2x10^6$/well in 24-well plates) were incubated with Concanavalin A (ConA, 2 μg/ml), various Neu peptides (10 μg/ml) or control gag peptide. Neu peptides were selected based on strong immunoreactivity *in vitro* with lymphocytes from BALB-*neu*T mice vaccinated with recombinant adenovirus expressing Neu or human ErbB2 receptor (15, Gallo *et al* unpublished data).

Mouse interferon-γ and IL-2 release into the supernatant was measured using an enzymatic immunocapture assay (Quantikine®, R&D Systems, Minneapolis MN). Results represent the mean of two independent experiments. Standard deviations were within 15% of the mean.

Statistical analysis. Statistical significance of prolonged median tumor-free survival and reduced median tumor incidence was determined by log-rank survival analysis using Prism4 GraphPad software (31). Differences in tumor infiltrating leukocyte number were evaluated by a two-tailed t-test.

Results

In vivo inhibition of neu oncogene-mediated mammary carcinogenesis by vaccination with normal rat Neu or human ErbB receptors. To compare tumor inhibitory properties of active immunization in *neu*-mediated tumorigenesis, female BALB-*neu*T mice were immunized with NIH3T3 fibroblasts overexpressing Neu (LTR-Neu) or human ErbB receptors including EGFR, ErbB2, ErbB3 or ErbB4. Vaccination was initiated at 6 weeks of age when mammary glands displayed atypical hyperplasia. By week 20, tumors had developed in all control mice subjected to mock treatment or immunization with NIH3T3. By contrast, none of the mice immunized with LTR-Neu or LTR-ErbB2 exhibited signs of tumor growth at this stage indicating specific interference with tumor development (Fig. 1). Subsequently, tumors began to form at week 22 and had affected all mice immunized with LTR-ErbB2 at week 27, whereas protective immunity of LTR-Neu persisted through week 31 in all animals. Moreover, at week 40 when tumor surveillance halted, 75% of animals in this group had remained tumor-free (Fig. 1). Overall, vaccination with LTR-Neu extended median tumor-free survival from 18 weeks in controls to >40 weeks ($p<0.0001$). By comparison, immunization with human ErbB orthologues significantly prolonged median tumor-free survival to 24.5 weeks for LTR-ErbB2 ($p<0.0001$), 21 weeks for LTR-EGFR ($p=0.001$) or LTR-ErbB3 ($p=0.002$) and 20 weeks for LTR-ErbB4 ($p=0.004$). Increased survival was paralleled by a concordant reduction in tumor prevalence. Vaccination delayed median

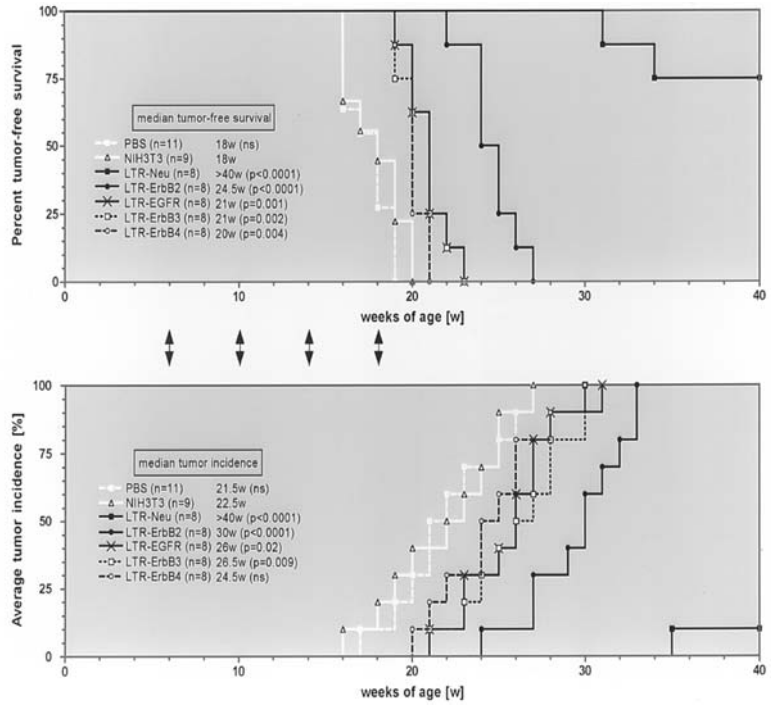

Figure 1. Inhibition of *neu* oncogene-mediated mammary carcinogenesis *in vivo* by vaccination with rat Neu or human ErbB2, EGFR, ErbB3 and ErbB4. Tumor-free survival (top) and average tumor incidence (bottom) in BALB-*neu*T mice vaccinated at the stage of atypical hyperplasia. Immunization schedule indicated by double arrowheads between panels at 6, 10, 14 and 18 weeks of age. Percent average tumor incidence was determined as cumulative number of tumors/number of animals using an end point of 10 tumors/mouse.

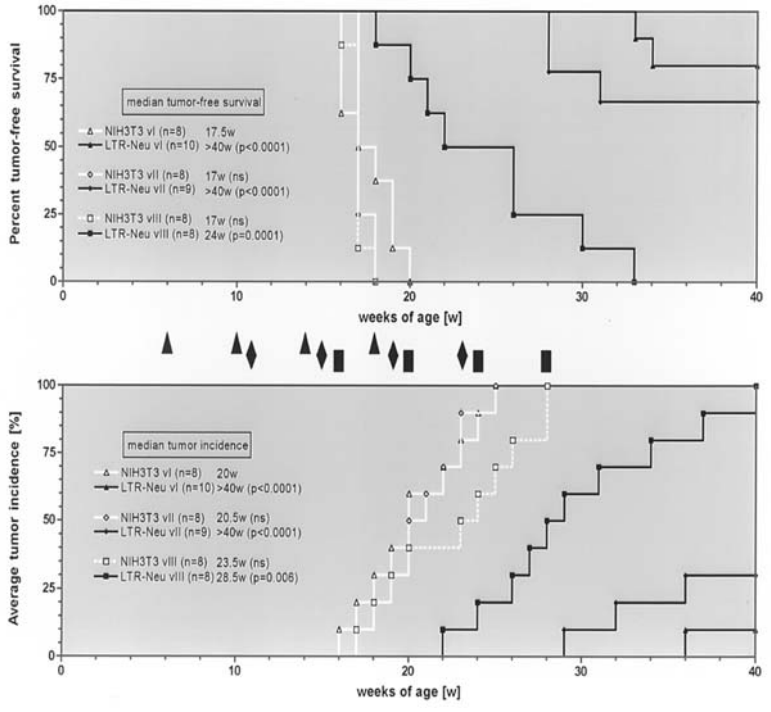

Figure 2. Inhibition of *neu*-induced mammary carcinogenesis by vaccination at different stages of tumor development. Tumor-free survival (top) and average tumor incidence (bottom) following vaccination by LTR-Neu at the stage of atypical hyperplasia (vI, triangle; 6, 10, 14, 18 weeks), *in situ* carcinoma (vII, diamond; 11, 15, 19, 23 weeks) and disseminated invasive carcinoma (vIII, box; 16, 20, 24, 28 weeks). Percent average tumor incidence was assessed as cumulative number of tumors/number of animals using an end point of 10 tumors/mouse.

tumor appearance from 22.5 weeks in controls to >40 weeks for LTR-Neu (p<0.0001), 30 weeks for LTR-ErbB2 (p<0.0001), 26.5 weeks for LTR-ErbB3 (p=0.009), 26 weeks for LTR-EGFR (p=0.02) and 24.5 weeks for LTR-ErbB4 (Fig. 1). These results demonstrated that interference with *neu*-mediated tumor growth in BALB-*neu*T mice was attained by active

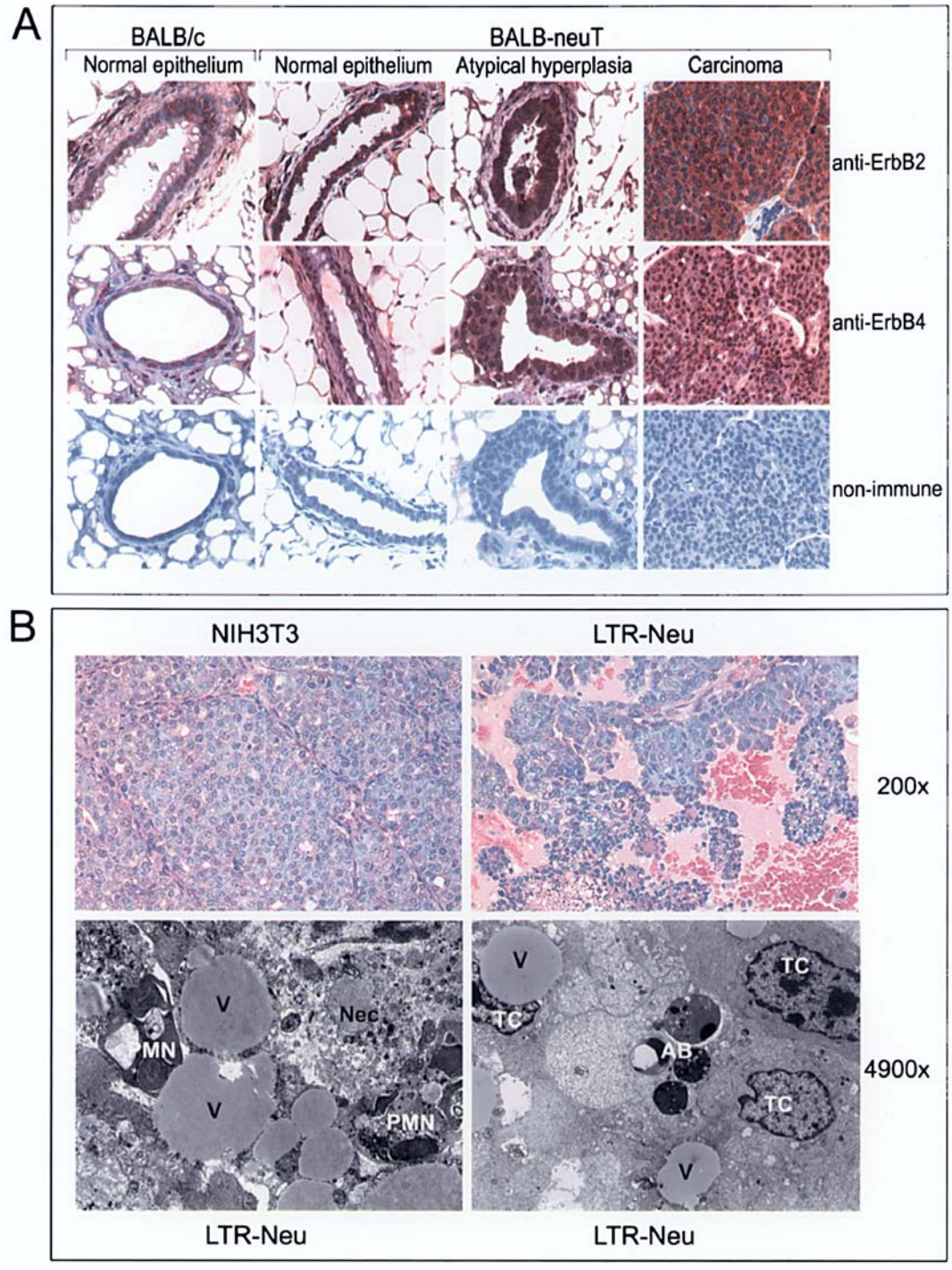

Figure 3. ErbB2/Neu and ErbB4 expression of glandular mammary epithelium and tumor necrosis in BALB*neu*T mice following LTR-Neu vaccination. (Panel A) Immunohistochemistry using ErbB2/Neu peptide antiserum M6, ErbB4 C-18 or rabbit non-immune serum in normal mammary glands of BALB/c and different stages of mammary tumor development of BALB-*neu*T. Immunoperoxidase counterstained with hematoxylin. Normal mammary glands of BALB/c and BALB-*neu*T were obtained at 5 weeks of age, while atypical hyperplasia or carcinoma tissue originate from mammary glands of BALB-*neu*T at 8 or 25 weeks of age, respectively. (Panel B) Histology (top) of BALB-*neu*T tumors upon vaccination with control NIH3T3 and LTR-Neu at the stage of atypical hyperplasia. Tumors of ~10-mm diameter were collected at 25 (NIH3T3) and 40 weeks (LTR-Neu) and stained using hematoxylin and eosin. Ultrastructural analysis (bottom) showing tumor necrosis (Nec) with free vacuoles (V) derived from dead tumor cells, polymorphonuclear granulocytes (PMN; A) and apoptotic bodies (B) in BALB-*neu*T mammary tumors following LTR-Neu vaccination at the stage of atypical hyperplasia. Original magnifications as indicated.

immunization with normal rat Neu as well as human ErbB receptors. It is noteworthy that the extent of tumor growth interference *in vivo* corresponded to the degree of overall conservation in predicted amino acid sequence with the rat Neu protein.

Early immunization favors vaccination efficiency. To determine whether tumor inhibition by vaccination depended on carcinogenesis stage, different immunization regimens were tested for LTR-Neu, starting at 6 (vI), 10 (vII) and 16 (vIII) weeks of age. As shown in Fig. 2, 80% or 67% of mice remained

Table I. Tumor infiltrating leukocytes (TIL) in BALB-*neu*T mice upon LTR-Neu or LTR-ErbB2 vaccination.

Tumor infiltrating leukocytes	BALB-*neu*T vaccination						
	6 weeks			11 weeks		16 weeks	
	NIH3T3	LTR-Neu	LTR-ErbB2	NIH3T3	LTR-Neu	NIH3T3	LTR-Neu
PMN, macrophages	11.2±3[a]	30.6±7 ($p=9.3x10^{-7}$)[b]	24.6±2 ($p=1.6x10^{-7}$)	12.9±3	31.5±3 ($p=5.8x10^{-7}$)	13±1.4	25.3±3 ($p=3.7x10^{-7}$)
B cells	7.1±1	7.7±1	8.1±1	8.9±1.9	8.6±2	9±1.3	8.4±2
$CD4^+$ lymphocytes	2.1±0.7	10.7±1.4 ($p=2.9x10^{-9}$)	8.1±1 ($p=4.6x10^{-7}$)	2.6±1.7	11.5±1.7 ($p=3.4x10^{-7}$)	3±1.8	8.2±2 ($p=8.5x10^{-6}$)
$CD8^+$ lymphocytes	1.8±0.7	11.9±2 ($p=2.9x10^{-7}$)	4.1±0.9 ($p=1x10^{-3}$)	2.3±1	8.1±1.9 ($p=3.2x10^{-6}$)	2.6±1.2	7.3±3 ($p=1.4x10^{-3}$)

[a]Cell count/field averaging 10 representative microscopic fields at x400 ± standard deviation. Leukocyte subpopulations were identified by immunohistochemistry. PMN, polymorphonuclear neutrophils. [b]Listing of p-value as determined by Student's t-test depicts significant difference in comparison with NIH3T3 controls.

tumor-free by week 40 when vaccination started at the stage of atypical hyperplasia (vI) or carcinoma *in situ* (vII), respectively. In contrast, later immunization at the stage of invasive carcinomas (vIII) yet still resulting in a significant delay of tumor growth was less effective with all mice exhibiting palpable tumors by week 33. Median tumor free-survival was more favorable in groups vI and vII (>40 weeks, $p<0.0001$) when compared with group vIII (24 weeks, $p=0.0001$) or NIH3T3 controls (17 weeks) exhibiting tumor formation in all mice at week 28 independent of immunization schedule (Fig. 2). Median tumor incidence was affected similarly with early immunization favouring more effective tumor suppression (Fig. 2). When immunization was initiated at the stage of atypical hyperplasia/carcinoma *in situ* (vI/vII), median tumor incidence was delayed from 20 or 20.5 weeks in NIH3T3 controls to >40 weeks ($p<0.0001$) as compared to a delay from 23.5 of NIH3T3 to 28.5 weeks ($p=0.006$) when immunization commenced at the carcinoma stage (vIII). These observations indicated that the tumor suppressive effect of vaccination was more effective the earlier immunization was initiated.

High ErbB2 and ErbB4 expression levels in glandular mammary epithelium of BALB-neuT mice and tumor necrosis following vaccination. ErbB receptor expression was assessed by immunohistochemistry in normal epithelium, atypical hyperplasia and carcinoma of BALB-*neu*T mice as compared to age-matched normal BALB/c controls. At 5 weeks of age, strong Neu/ErbB2- and ErbB4-specific staining was confined to glandular epithelial cells of normal BALB/c mice (Fig. 3A), while EGFR and ErbB3 expression was not detectable above non-immune controls (Fig. 3A and data not shown). These findings are consistent with a physiologic role of endogenous Neu/ErbB2 and ErbB4 at this stage of normal mammary gland development or homeostasis, preceding tumor onset in BALB-neuT mice. By comparison, ErbB2 and ErbB4 staining was more intense and homogeneous in normal mammary epithelium of *neu* transgenic mice at this stage. Both ErbB2 and ErbB4 expression persisted at high levels through the subsequent phases of atypical hyperplasia and neoplasia in BALB-*neu*T mice. In contrast to Neu expression localizing to cytoplasm and cell membrane, however, ErbB4 expression was distinctly nuclear in addition to its cytoplasmic detection (Fig. 3A). Although a functional role of nuclear ErbB4 expression in the context of oncogenic *neu* activation has not been established, it is noteworthy that spontaneous human malignancies with etiologic ErbB receptor involvement including human breast cancer exhibit nuclear ErbB4 overexpression (32). While enhanced Neu expression in BALB*neu*T mammary epithelium conforms with detection of transgenic *neu* product in addition to endogenous normal Neu protein, increased ErbB4 expression indicates direct or indirect upregulation of endogenous expression resulting from *neu* oncogene function. Occurrence in normal epithelium of BALB*neu*T suggests elevated Neu and ErbB4 expression to precede tumor formation (Fig. 3A). Expression level and localization of Neu was comparable in normal epithelium, atypical hyperplasia and invasive carcinoma from all vaccination groups of BALB-*neu*T mice (data not shown) indicating that tumor inhibition by vaccination was not effected by overall down-regulation of Neu protein expression of tumors.

Incidence of invasive mammary carcinoma affected all glands from BALB-*neu*T mice subjected to control immunization using NIH3T3 (Fig. 3B). Conversely, palpable tumors from LTR-Neu immunized mice harbored large areas of ischemic and hemorrhagic necrosis characterized by cellular detachment and vacuolization with extravasation of erythrocytes and edema (Fig. 3B). On the other hand, tumors from LTR-ErbB2- or at a later stage LTR-Neu-vaccinated mice displayed only focal areas of ischemic and hemorrhagic necrosis at the tumor margins (data not shown). Tumors from mice vaccinated early with LTR-Neu showed vacuolization of tumor cells, cellular debris, necrotic cells and polymorphonuclear granulocytes infiltrating the necrotic areas (Fig. 3B). Furthermore, apoptotic bodies were discerned within the

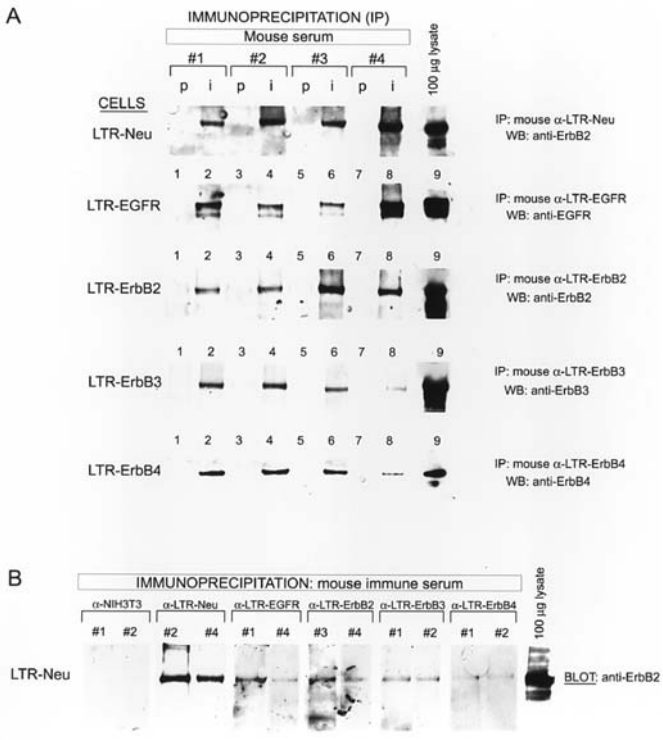

Figure 4. Serum antibody response of mice upon vaccination with rat Neu or human ErbB receptors. (A) Induction of serum antibodies specific for the authentic immunogen following vaccination at the stage of mammary atypical hyperplasia. Sera from 4 different animals/immunization group (#1-#4) were collected, at 6 weeks (lane 1, 3, 5 and 7; p=preimmune) and 19 weeks of age (lane 2, 4, 6 and 8; i=immune). Sera were used in immunoprecipitation of the immunogen from the respective LTR-ErbB transfectant (500 μg lysate). ErbB receptor specificity was visualized by immunoblot analysis using receptor-specific polyclonal peptide antisera of immunoprecipitates and compared to direct immunoblotting of 100 μg LTR-ErbB lysate (lane 9) as positive control. (B) Crossreactivity with rat Neu of immune sera elicited by vaccination with LTR-EGFR, -ErbB2, -ErbB3 and -ErbB4 receptors, respectively. Numbering identifies immune sera of individual mice, which were collected one week after the final boost (19 weeks). Immune sera of LTR-Neu (#3 and #4) and NIH3T3 (#1 and #2) vaccinated mice served as positive and negative controls, respectively.

tumor mass (Fig. 3B). Tumor infiltrating leukocytes (TIL) were differentiated by immunohistochemistry in BALB-*neu*T mice upon LTR-Neu or LTR-ErbB2 vaccination (Table I). Successful vaccination using LTR-Neu or LTR-ErbB2 was accompanied by significant changes in composition of tumor-infiltrating leukocytes (Table I). In comparison to NIH3T3 controls, segmented neutrophils, macrophages, CD4- and CD8-positive T cells increased upon LTR-Neu immunization initiated at 6, 11 and 16 weeks and upon LTR-ErbB2 vaccination at the hyperplastic stage, while there was no significant change in B cell number (Table I).

B cell immunity following LTR-Neu and LTR-ErbB vaccination. A specific antibody response to individual ErbB receptors was qualitatively and quantitatively investigated by immunoblotting or ELISA, respectively. Mouse pre-immune or immune sera were collected prior to vaccination or one week after the final boost, respectively and utilized in immunoprecipitation of the corresponding ErbB receptor from recombinant LTR-ErbB transfectants. Specific reactivity was visualized by immunoblotting of immunoprecipitates using receptor-specific peptide antisera. Analysis of 4 representative animals in each immunization cohort is depicted in Fig. 4A. Specific antibodies were detected in all mice vaccinated with LTR-Neu or individual LTR-ErbB receptors. Specific antibody titers to individual immunogens were quantitated by ELISA. As shown in Table II, immunization with all LTR-transfectants produced high IgG titers specific for the corresponding ErbB immunogen exceeding 10^{-3}. By comparison, serum titers to individual immunogens were of the same order of magnitude including LTR-Neu (1:2700), LTR-EGFR (1:3200), LTR-ErbB2 (1:3400), LTR-ErbB3 (1:2300) or LTR-ErbB4 (1:2400) suggesting comparable immunization efficiency for various immunogens.

The extent of cross-reactivity with the etiologic *neu* oncogene product of immune sera following vaccination with human ErbB receptors was assessed by immunoprecipitation

Table II. Immunogenicity of individual ErbB receptors in BALB-*neu*T and immunoreactivity of mouse sera with rat Neu extracellular domain (Neu-ECD).

Immunogen	Antigen[a]		Immunoglobulin isotype against Neu-ECD					
	LTR-ErbB	Neu-ECD	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
PBS	0/8[b] (Neg)	0/8 (Neg)	nd	nd	nd	nd	nd	nd
NIH3T3	nd[c]	0/16 (Neg)	nd	nd	nd	nd	nd	nd
LTR-Neu	16/16 (1:2700)[d]	16/16 (1:4000)[e]	12.5[f] (±0.2)	21.8 (±0.2)	23 (±0.3)	25.7 (±0.6)	9.45 (±0.9)	7.5 (±0.2)
LTR-EGFR	11/11 (1:3200)	11/11 (1:150)	9.45 (±2)	22 (±2)	19.8 (±0.9)	23.7 (±0.3)	14 (±2)	10 (±0.9)
LTR-ErbB2	11/11 (1:3400)	11/11 (1:725)	9.25 (±2)	21.9 (±2)	19.7 (±1)	23.9 (±0.7)	14.3 (±2)	10.5 (±0.9)
LTR-ErbB3	11/11 (1:2300)	11/11 (1:100)	10.6 (±3)	20.7 (±2)	18.5 (±1)	24.8 (±0.4)	15 (±0.2)	9.8 (±0.2)
LTR-ErbB4	11/11 (1:2200)	11/11 (1:100)	10.8 (±2.8)	20.6 (±2)	18.4 (±0.8)	24.6 (±0.2)	15 (±0.6)	10.3 (±0.4)

[a]Immune serum titer of BALB-*neu*T vaccinated mice were determined by ELISA against the respective immunogen (LTR-ErbB transfectant: LTR-Neu, LTR-EGFR, LTR-ErbB2, LTR-ErbB3, LTR-ErbB4) or recombinant Neu-ECD; [b]number of mice with immune response/total; [c]not determined; [d]titer was estimated as the highest immune serum dilution generating a specific absorbance of 2.0 at 492 nm under conditions, in which optical density of all immune sera at 1:200 to NIH3T3 controls was <0.5; [e]highest mouse immune serum dilution sufficient to yield absorbance of 0.5 at 492 nm, whereas OD492 of all immune sera at 1:100 with BSA was <0.05; [f]percent distribution (± standard deviation) of specific immunoglobulin isotypes reactive with recombinant Neu extracellular domain.

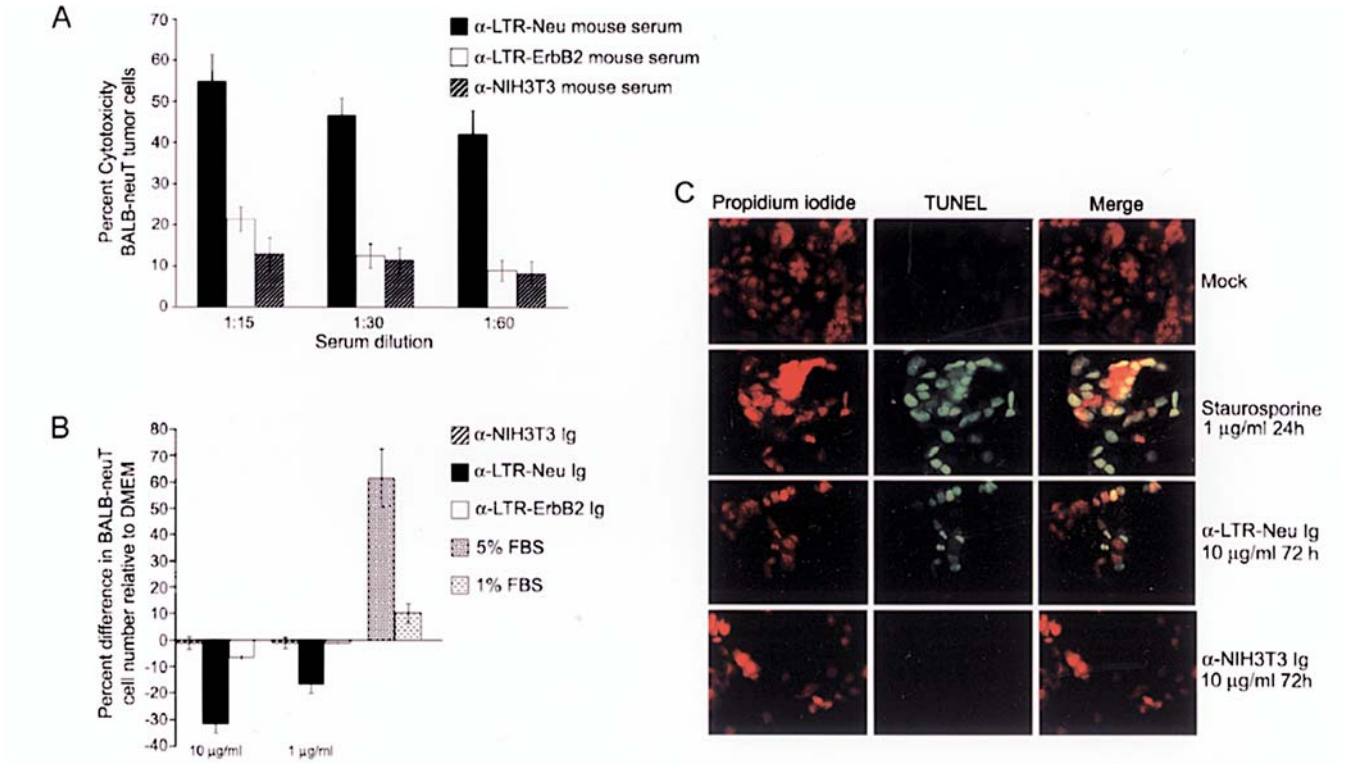

Figure 5. Biological activity *in vitro* of immune sera or purified immunoglobulins of LTR-Neu or LTR-ErbB2 vaccinated mice. (A) Specific antibody-dependent cell-mediated cytotoxicity elicited by vaccination at the stage of atypical mammary hyperplasia. BALB-*neu*T mammary cancer cells were exposed for 2 h to sera pooled from LTR-Neu, LTR-ErbB2 and NIH3T3 vaccinated mice at different dilutions and following washes co-cultivated at 37˚C overnight with mononuclear effector cells derived from normal BALB/c spleens at a ratio of 50:1. Results represent average percent cytotoxicity of two independent experiments. (B) Proliferation of BALB-*neu*T mammary tumor cells upon exposure to purified serum immunoglobulins (Ig) from LTR-Neu, LTR-ErbB2 and NIH3T3 mice vaccinated at the stage of atypical mammary hyperplasia. BALB-*neu*T mammary cancer cells following serum depletion were incubated in DMEM medium containing 0.2% BSA with or without purified Ig (10 μg/ml or 1 μg/ml chronically added each 24 h) and compared to growth medium containing 1% or 5% FBS. Relative cell numbers of triplicate experiments were determined after incubation of 72 h at 37˚C using a sulforhodamine B based proliferation assay and expressed as percent increase or decrease in comparison to vehicle control (DMEM 0.2% BSA). (C) Induction of apoptosis by anti-LTR-Neu immunoglobulins in BALB-*neu*T mammary tumor cells. Cells were plated at $5x10^3$ cells/well in chamber slides, incubated in DMEM medium containing 0.2% BSA with or without purified Ig (10 μg/ml added every 24 h). Staurosporine (1 μM) treatment of 24 h was used as positive control.

of LTR-Neu lysates using individual mouse sera from mice vaccinated with LTR-EGFR, LTRErbB2, LTR-ErbB3 or LTR-ErbB4 followed by immunoblotting with an ErbB2-specific peptide antiserum. As shown in Fig. 4B, serum antibodies induced upon vaccination with human ErbB receptors exhibited variable cross-reactivity with rat Neu. Cross-reactivity was quantitated by ELISA using recombinant rat Neu extracellular domain (Neu-ECD) as antigen. Mice vaccinated with LTR-Neu developed a high titer antibody response to Neu extracellular domain (1:4000). An anti-Neu-ECD antibody response was comparably high even at latter vaccination onset. Anti-Neu titers of 1:4000, 1:3500 or 1:3250 were determined with vaccination regimens initiated at 6, 11 or 16 weeks, respectively. By comparison, specific immunity with Neu-ECD was weaker in mice vaccinated with human ErbB receptors including LTR-ErbB2 (1:725), LTR-EGFR (1:150), LTR-ErbB3 (1:100) and LTR-ErbB4 (1:100) reflecting a varying degree of cross-reactivity with Neu-ECD (Table II).

There was no pronounced shift in distribution of Neu-ECD specific immunoglobulin subclasses with IgG2a/2b and IgG3 populations representing the most prevalent immunoglobulin subtypes in all vaccination cohorts (Table II). Thus, titer differences reflected relative avidity of immune sera for Neu extracellular domain.

In an attempt to explore possible mechanisms of tumor inhibition, antibody-dependent cellular cytotoxicity (ADCC) and cell proliferation of BALB-*neu*T mammary tumor cells was analyzed using purified immunoglobulins (Ig) of mice immunized at 6 weeks of age. Pooled sera from LTR-Neu-vaccinated mice mediated ADCC of BALB-*neu*T tumor cells upon short-term exposure of 2 h at dilutions up to 1:60, while anti-LTR-ErbB2 mouse sera were less effective in their ability to elicit ADCC even at a higher concentration of 1:15. Furthermore, chronic treatment for 72 h using purified Ig from LTR-Neu-vaccinated mice inhibited cell proliferation by 30% at a concentration of 10 μg/ml relative to vehicle or anti-NIH3T3 controls (Fig. 5B). Specific inhibition of cell growth was also discerned at 1 μg/ml Ig, whereas anti-LTR-ErbB2 immunoglobulins reduced cell proliferation by 6% only at 10 μg/ml (Fig. 5B). To determine if specific immunoglobulins were capable of inducing apoptosis, BALB-*neu*T tumor cells were stained by TUNEL labeling upon chronic treatment with anti-LTR-Neu or anti-NIH3T3 immunoglobulins at 10 μg/ml. The fraction of apoptotic cells was estimated relative to propidium iodide-positive cells. In comparison to 1 μg/ml staurosporine treatment resulting in 80% apoptotic cells, purified Ig from LTR-Neu-vaccinated mice mediated apoptosis of approximately 20% of BALB-*neu*T tumor cells,

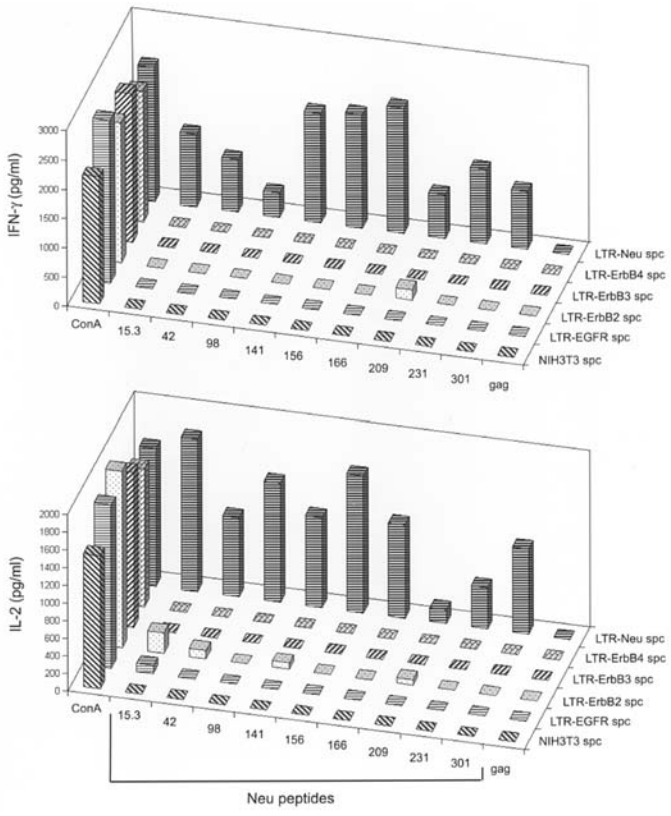

Figure 6. T-cell immune response of BALB-*neu*T mice following vaccination with rat Neu or human ErbB receptors. Spleen cells (spc) from mice vaccinated at the stage of atypical mammary hyperplasia including LTR-Neu, LTR-EGFR, LTR-ErbB2, LTR-ErbB3, LTR-ErbB4 and NIH3T3, were stimulated *in vitro* with Neu-specific peptides derived from ECD (Neu 15.3, aa 66-74; Neu 42, aa 169-183; Neu 98, aa 393-407; Neu 141, aa 566-580; Neu 156, aa 626-640), TMD (Neu 166, aa 666-680) and ICD (Neu 209, aa 838-852; Neu 231, aa 926-940; Neu 301, aa 1206-1220) and selected according to *in vitro* immunoreactivity with lymphocytes from BALB-*neu*T mice vaccinated with recombinant adenovirus expressing Neu or human ErbB2 (15, Gallo *et al* unpublished data). Release of IFN-γ and IL-2 was quantitated in the supernatant as a measure of T cell immunoreactivity with specific Neu epitopes. ConcanavalinA for global T cell activation and an unrelated gag peptide served as positive and negative control, respectively.

while a similar effect of anti-LTR-ErbB2 was not detectable (Fig. 5C). Thus, *in vitro* biologic activity including inhibition of cell proliferation and induction of apoptosis by distinct immune sera following vaccination corresponded to their ability of interfering with tumor growth *in vivo*.

T-cell immune response by LTR-Neu and LTR-ErbB receptor vaccination. To evaluate Neu T cell immunity elicited by LTR-Neu or LTR-ErbB immunization, IFN-γ and IL-2 release was measured following triggering of spleen cells from vaccinated mice with Neu-specific epitopes. Synthetic peptides were selected based upon known immunogenicity *in vitro* for lymphocytes of BALB-*neu*T mice vaccinated with recombinant adenovirus expressing Neu or human ErbB2 (15, Gallo *et al* unpublished data). All Neu peptides utilized but not an unrelated gag peptide were able to specifically activate spleen T cells from BALB-*neu*T mice vaccinated with LTR-Neu at the stage of atypical mammary hyperplasia as measured by release of IFN-γ (top panel) or IL-2 (bottom panel). When compared to global T cell stimulation by Concanavalin A (ConA), cytokine response was robust for certain Neu epitopes. Although release of both cytokines was triggered by all Neu peptides in lymphocytes of LTR-Neu-vaccinated mice, the level of cytokine secretion was heterogeneous between IFNγ and IL-2 (Fig. 6). Moreover, a weaker Neu-specific T cell response was suggested by the ability of certain Neu peptides to elicit cytokine release from lymphocytes of mice vaccinated with LTR-ErbB2 or LTR-EGFR but not NIH3T3 control or the other human ErbB receptors (Fig. 6). In LTR-ErbB2-vaccinated mice, a Neu-specific T cell response was detected for peptide 209 in the tyrosine kinase domain prompting IFNγ and IL-2 release or peptides 15.3, 42, and 141 in the extracellular domain by IL-2 release. Furthermore, weak T cell immunity to Neu epitope 15.3 in the extracellular domain was also observed in mice vaccinated with LTR-EGFR (Fig. 6). These findings suggested a robust Neu-specific T cell response in BALB-*neu*T mice following LTR-Neu vaccination. Apparently, a weaker T cell response to some of these epitopes may also be recruited by immunization with human ErbB receptors, which might depend on the extent of sequence conservation of individual peptide epitopes with the Neu protein.

Discussion

Findings of this study indicated gene-specific interference with *neu* mammary tumorigenesis in the transgenic BALB-*neu*T mouse model following active immunization with rat LTR-Neu as well as human LTR-ErbB2, LTR-EGFR, LTR-ErbB3 and LTR-ErbB4 in descending order of an inhibitory response. Thus, cross-vaccination efficiency by immunization with human ErbB receptors overall appeared to correspond to relative sequence conservation with the rat Neu product. The most pronounced regression of tumor growth *in vivo* was associated with detection of specific antibody and cellular immunity, resulting in growth inhibition and apoptosis of tumor cells *in vitro*. Clinical studies have established efficacy of recombinant humanized monoclonal antibody (trastuzumab) recognizing the ECD of the ErbB2 protein to induce an objective response in breast cancer patients (33-35). These studies, however, have also indicated that an objective response to trastuzumab monotherapy had a median duration of 9 months, and that the majority of responsive patients developed resistance within 1 year (35). These studies further demonstrated that cancer patients greatly benefited from combination of Mab-based immunotherapy with chemotherapy. In light of these observations it is reasonable to explore alternative immunotargeting approaches with the rationale of improving an objective tumor inhibitory response (36). Active immunization targeting ErbB2 might sustain tumor inhibition more effectively than passive immunotherapy based on the induction of a sustained memory immune response. It would also be applicable in boosting a naturally occurring ErbB2 immune response. Moreover, active immunization using ErbB2 as immunogen might be advantageous to a single Mab by simultaneously eliciting T and B cell immunity to multiple immunodominant epitopes. This possibility is supported by observations indicating that a combination of therapeutic Mabs trastuzumab and pertuzumab binding to distinct epitopes of ErbB2 oligomers is more effective in

suppressing proliferation and survival of ErbB2-positive human breast cancer cells (37). Naturally, there are safety concerns in vaccination involving a potent oncogene. Since oncogenic ErbB2 function, however, is strictly dependent on its intrinsic tyrosine kinase activity, attenuation by mutation of its kinase domain might be a viable alternative.

Among active immunotherapy approaches, vaccination using a xenogeneic orthologue has previously been shown effective in eliciting cross-reactive immunity and antitumor activity to homologous antigens (38-43). We demonstrated that interference with *neu*-mediated tumor growth in BALB-*neu*T mice was achieved by active immunization using normal rat Neu as well as human ErbB receptors. The relative extent of tumor growth interference *in vivo,* paralleled by the degree of overall predicted amino acid sequence conservation with the rat Neu protein, was also reflected in relative titers of anti-Neu-ECD serum antibodies elicited by vaccination with distinct human ErbB receptor homologues. There was evidence for the existence of serum antibodies cross-reacting with the Neu protein following immunization with each human ErbB receptor. Furthermore, a cross-reactive T cell response to defined Neu epitopes was detected following vaccination with LTR-ErbB2 and LTR-EGFR, but not LTR-ErbB3 and LTR-ErbB4. A heterologous T cell response might yet have been elicited by neuregulin receptors for other Neu epitopes not tested here. Biologic relevance *in vivo* of immunocompetent epitopes present in the four ErbB receptors was supported by significant interference with *neu*-mediated mammary carcinogenesis resulting from vaccination with each of the four human ErbB receptors. In case of LTR-EGFR or LTR-ErbB3 vaccination, protracted tumor-free survival was achieved although endogenous expression of mouse orthologues was undetectable in mammary epithelial cells at the stage of tumor onset or progression. Since immunization with each human ErbB receptor resulted in comparably high specific antibody titers to the autologous immunogen, varying immunization efficiency cannot account for differences in tumor growth inhibition. Conversely, recruitment of immunodominant epitopes shared with the etiologic neu oncoprotein might be responsible for the biological effect. Identification of cross-reactive immunogenic epitopes among different ErbB receptors would facilitate concurrent targeting of multiple ErbB receptors *in vivo*. The significance of such a notion is underlined by observations that ErbB receptors, foremost ErbB2 and ErbB3, potently synergize by heterodimerization in mitogenicity, oncogenic transformation and human cancer (1).

Physiologic, endogenous ErbB2 and ErbB4 expression of mammary epithelial cells was robust in normal BALB/c mice and upregulated in normal epithelium of BALB-*neu*T at a phase preceding tumor development. Intriguingly, ErbB4 exhibited nuclear localization. Nuclear ErbB4 expression has been observed in various tumors including breast cancer and functionally linked to regulated intramembrane proteolysis (RIP) ensued by nuclear translocation of ErbB4 intracellular domain polypeptides (32,44). Whether unique post-translational processing of ErbB4 is associated with a distinct biologic phenotype or is a general property of ErbB4 function resulting in direct activation of transcriptional targets has not been resolved. Using different genetic approaches, ErbB4 has been shown to be essential in physiological, lobuloalveolar terminal differentiation and lactogenesis of mammary epithelium (45,46). On the other hand, ErbB4 loss of function does not alter incidence or latency of MMTV-driven *neu* mammary tumorigenesis (47) indicating that the nuclear ErbB4 expression associated with tumorigenesis in BALB-*neu*T mice shown here might reflect a discrete differentiation stage of mammary epithelial cells rather than exerting oncogenic properties in *neu*-mediated mammary carcinogenesis. LTR-ErbB4 vaccination among human ErbB receptors was least efficient in suspending *neu*-mediated tumor growth in BALB-*neu*T mice. Though vaccination resulted in a high specific serum antibody titer of 1:2,200 comparable to those of other ErbB receptors, it remains elusive whether unique proteolytic processing resulting in rapid release of its extracellular domain contributes to a lower efficiency in recruiting immunodominant B cell or T cell epitopes cross-reactive with oncogenic *neu*.

Vaccination with rat Neu in an allogenic cell background has previously been shown to be effective in reduction of tumor formation in the BALB-*neu*T transgenic model (48). Furthermore, inhibition of carcinogenesis in BALB-*neu*T has required a high titer of specific anti-Neu antibodies (13,16,17). Our observations indicated that tumor suppression was more effective in case of an earlier immunization onset. Distinct mechanisms including ADCC, CDC (complement dependent cytotoxicity), induction of apoptosis, or inhibition of receptor heterodimerization have been implicated to rationalize the inhibitory effect of anti-ErbB2/Neu antibodies on the growth of cancer cells expressing ErbB2/Neu (16,17,49-54). Here we showed that immune sera from LTR-Neu-vaccinated mice inhibited cell proliferation, mediated ADCC and induced apoptosis of BALB-*neu*T tumor cells *in vitro*, which corresponded to the relative extent of a tumor inhibitory effect *in vivo*. However, regression of established tumors following vaccination was less effective presumably due to insufficient antibody accessibility or higher antibody requirement. Synergy of B and T cell immunity is essential for eradication of Neu expressing tumors (55). Successful vaccination using LTR-Neu or LTR-ErbB2 was reflected in significant changes of tumor-infiltrating leukocyte composition (Table I). TIL might be directly involved in tumor rejection. In conjunction with anti-Neu antibodies, they might locally facilitate tumor cell elimination or produce cytokines with antiangiogenetic properties, which mediate ischemic necrosis at the tumor site (56). Among others, IFN-γ has been established in tumor rejection by deprivation of blood supply (57). We demonstrated that spleen T cells of vaccinated mice were primed for secretion of IFN-γ and IL-2 upon stimulation with several Neu-specific peptides. Recognition of these epitopes *in vivo* potentially activates T cells to produce IFN-γ thus causing ischemic necrosis at the tumor site. Such immunodominant epitopes are candidates for boosting an immune response in BALB-*neu*T mice. In addition, cross-vaccination by multiple human ErbB receptors facilitates identification of immunodominant epitopes with potential relevance for immunotherapy of human tumors.

Eventual tumor penetration in *neu* transgenic mice in spite of an active anti-Neu immune response has also been reported by others (13,17,58). Antigen loss does not appear to account for tumor growth in LTR-Neu-vaccinated mice as Neu protein expression levels were similar in tumors from immunized and control BALB-*neu*T mice. In this context, there are several

reports indicating that ErbB2 might not be consistently down-regulated following trastuzumab treatment (35). On the other hand, immunosuppressive factors released by tumor cells or alternatively modulation of regulatory T cells (Treg) might compete with sustained T cell-mediated antitumoral immunity (59-61). Our results indicate that efficient inhibition of *neu* oncogene-mediated mammary carcinogenesis by active immunization depends both on the carcinogenesis stage at which vaccination is initiated as well as recruitment of specific immunogenic epitopes. Since several of these epitopes are recruited by immunization with human ErbB receptors it appears worthwhile to investigate whether targeting of such epitopes inhibits growth of human malignancies.

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