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Attenuation of PI3K/Akt-Mediated Tumorigenic Signals through PTEN Activation by DNA Vaccine-Induced Anti-ErbB2 Antibodies

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By studying BALB/c mice deficient in immune components, we show that the protective immunity to rat ErbB2⁺ tumors rests on the Ab response elicited by the electroporation of a DNA vaccine encoding the extracellular and transmembrane domains of rat ErbB2. In vivo, the adoptive transfer of vaccine-elicited anti-rat ErbB2 Abs protected against a challenge of rat ErbB2⁺ carcinoma cells (TUBO cells). In vitro, such Abs inhibited TUBO cell growth by impairing cell cycle progression and inducing apoptosis. To correlate intrinsic mechanisms of Ab action with their tumor-inhibitory potential, first we showed that TUBO cells constitutively express phosphorylated transgenic ErbB2/autochthonous ErbB3 heterodimers and exhibit a basal level of Akt phosphorylation, suggesting a constitutive activation of the PI3K/Akt pathway. Treatment with anti-ErbB2 Abs caused a drastic reduction in the basal level of Akt phosphorylation in the absence of an impairment of PI3K enzymatic activity. Notably, the same Ab treatment induced an increase in PTEN phosphatase activity that correlated with a reduced PTEN phosphorylation. In conclusion, vaccine-induced anti-ErbB2 Abs directly affected the transformed phenotype of rat ErbB2⁺ tumors by impairing ErbB2-mediated PI3K/Akt signaling. *The Journal of Immunology*, 2010, 184: 000–000.

The ErbB2 protein (HER2/neu in humans and neu in rat) belongs to a family of receptor tyrosine kinases that include four members (ErbB1–4) with distinct recognition specificities for multiple ligands of the epidermal growth factor superfamily (1, 2). ErbB2 signaling involves ligand-induced receptor homo- and heterodimerization in which ErbB2 acts as a ligand-less receptor recruited into heterodimers with other ErbB members (1). The resultant ErbB2-containing heterodimers are characterized by a diversified and remarkable superiority in signaling capability, resulting in a more intense and prolonged signal output (3, 4).

Amplification of the *ErbB2* gene occurs in 20–30% of human breast cancers and is linked to a more aggressive disease course and worse prognosis (5–7). In ErbB2⁺ tumor cells, the receptor can function on its own and/or it needs to heterodimerize with

another ErbB member to transduce a deregulated proliferative signal responsible for the neoplastic behavior of the cells (8–10).

A pivotal signaling pathway for tumorigenesis is the PI3K/Akt pathway, which controls growth, survival, and proliferation and is regulated by the antagonistic activities of PI3K and the tumor suppressor phosphatase and tensin homolog deleted from chromosome 10 (PTEN). PI3Ks are proto-oncogenic enzymes that catalyze the production of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP₃) (11). In contrast, PTEN phosphatase activity counteracts PI3K activity, dephosphorylating the 3-position of PIP₃ (12, 13). The transient accumulation of PIP₃ at specific plasma membrane compartments triggers the recruitment and activation of protein kinase effectors, such as protein kinase B/Akt, which ultimately lead to proliferative and survival cell responses.

The PI3K/Akt pathway is widely activated in ErbB2-overexpressing breast tumors. However, the potency and kinetics of PI3K activity depend on the composition of the receptor pair: ErbB1 and ErbB2 do not have direct binding sites for PI3K, whereas ErbB3 has six PI3K-binding sites (14, 15). Thus, the ErbB2/ErbB3 heterodimer is the most potent activator of the PI3K/Akt pathway (10, 16).

The oncogenic potential of ErbB2 and its accessibility make this receptor an attractive target for therapeutic strategies based on the use of specific mAbs. mAbs directed against the extracellular (EC) domain of the rodent or human version of wild-type or mutated ErbB2 proteins were shown to confer inhibitory effects on tumor growth in vivo and in vitro (17–19). Trastuzumab (or Herceptin), a humanized murine Ab against human ErbB2, significantly improved clinical outcomes of patients with ErbB2-overexpressing breast cancer (20–22). However, the efficacy of trastuzumab in the long-term treatment of these patients is challenged by the observation that up to 70% of them eventually develop resistance to trastuzumab (23).

To reduce the risk for tumor escape, a promising strategy consists of the use of a mixture of mAbs to multiple epitopes of the ErbB2R. Previous studies demonstrated a cooperative and even synergic

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Abbreviations used in this paper: EC, extracellular; EC-TM^{neu}, neu EC and TM domains; HRG, heregulin; pAb, polyclonal Ab; pAkt, phospho-Akt; PI, propidium iodide; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; PTEN, phosphatase and tensin homolog deleted from chromosome 10; RT, room temperature; TM, transmembrane.

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effect of Ab combination in terms of antitumor potential (24–30). Therefore, an immunization protocol aimed at generating long-lasting Ab response against ErbB2⁺ carcinomas may be effective because of its ability to elicit multiple Ab-mediated mechanisms of protection and to provide a prolonged therapeutic benefit.

We showed that vaccination of mice with a plasmid encoding for the EC and transmembrane (TM) domains of rat ErbB2 (EC-TM^{neu}) protects from a lethal challenge of ErbB2⁺ tumor cells by eliciting a cell-mediated and a humoral immune response (31, 32). EC-TM^{neu} vaccination also leads to the inhibition of spontaneous mammary tumors in rat ErbB2 transgenic mice, mainly by eliciting anti-rat ErbB2 Abs capable of inhibiting the expansion of ErbB2⁺ cells in vitro (33, 34). However, the molecular mechanisms underlying Ab-induced retardation/rejection of tumor growth have not been elucidated.

Materials and Methods

Mice

Seven-week-old wild-type BALB/c mice were from Charles River Laboratories (Calco, Italy); BALB/c mice knocked-out for the *Ig* μ -chain gene (BALB- μ IgKO) and for the *pfp* gene (BALB-pfpKO) were provided by Dr. T. Blankenstein [University of Berlin, Berlin, Germany; (35)] and Dr. Mark J. Smyth [Peter MacCallum Cancer Centre, East Melbourne, Australia; (36)], respectively. Mice were treated properly and humanely in accordance with the European Union guidelines.

Cell lines and reagents

TUBO cells were generated from a mammary carcinoma arising in a BALB/c female mouse transgenic for the activated rat ErbB2 oncogene (BALB-neuT mouse) (31); BALB/c 3T3 fibroblasts stably transfected with the wild-type rat ErbB2 (3T3/NKB) were provided by Dr. W. Z. Wei (Karmanos Cancer Institute, Detroit, MI) (37).

The rabbit anti-rat EGFR/ErbB1, ErbB2 (C-18), ErbB3 (C-17), and ErbB4 (C-18) polyclonal Abs (pAbs) and the mouse anti-PTEN (A2B1) mAb were from Santa Cruz Biotechnology (Santa Cruz, CA); the mouse anti-rat ErbB2 (B10) mAb was from Lab Vision (Thermo Fisher Scientific, Fremont, CA); the mouse anti-rat ErbB2 (Ab-4) mAb was from Calbiochem (Darmstadt, Germany); the mouse anti-phosphotyrosine (4G10) mAb and the rabbit anti-PI3K p85 pAb were from Upstate Biotechnology (Lake Placid, NY); and the rabbit anti-Akt and anti-phospho-Akt (Ser473) pAbs were from Cell Signaling Technology (Beverly, MA).

The recombinant rat full-length ErbB2 protein was from GenWay Biotech (San Diego, CA). Anti-MHC class I H-2K^d mAb (31-3-4S) was from Cedarlane Laboratories (Hornby, Ontario, Canada). The recombinant heregulin (HRG)- β 3 was a gift from Prof. M. Alimandi (Sapienza University). pcDNA3 empty vector and vector coding the EC and TM domains of the rat ErbB2 receptor (EC-TM^{neu}) were produced and used as previously described (31).

Vaccination of mice

The vaccination was performed by i.m. DNA electroporation as we previously described in detail (38). Each course of DNA vaccination consisted of two electroporations with an interval of 7 d. Mice of the same age were randomly assigned to the control and treatment groups, and all groups were treated concurrently. Sera were collected 14 and 28 d after the last immunization. Sera from the same treatment group were pooled and stored at 4°C.

Tumor challenge and evaluation of tumor growth

BALB/c, BALB- μ IgKO, and BALB-pfpKO mice were challenged s.c. in the right flank with 0.2 ml suspension containing the minimal lethal dose of TUBO cells (10^5). Neoplastic masses were measured with calipers, and tumor size was expressed as the mean of the two perpendicular diameters. Progressively growing masses >1 mm in mean diameter were regarded as tumors. Mice were first vaccinated when they presented a palpable tumor mass 2 mm in mean diameter, boosted 7 d later, and tumor growth was followed for 75 d; subsequently, tumor-free mice were classified as survivors. Tumor-bearing mice were killed for humane reasons when the tumor exceeded 10 mm in mean diameter.

Evaluation of ErbB2-binding activity of purified IgG

Total IgG was purified from the sera of mice immunized with empty vector (control IgG) or vector coding EC-TM^{neu} (immune IgG) using an ImmunoPure (A Plus) IgG Purification kit (Pierce, Rockford, IL); the presence of IgG specific for ErbB2 was evaluated by cytofluorometric analysis before and after ErbB2 neutralization and by ELISA using a recombinant rat ErbB2 protein.

To neutralize the IgG specific for EC-TM^{neu}, pools of IgG purified from control or immune sera were incubated with a large excess of recombinant rat ErbB2 protein (1:4 ratio) for 1 h at room temperature (RT). To evaluate the ability of purified IgG to bind rat ErbB2, TUBO (5×10^5) and 3T3/NKB cells (5×10^5) were stained in a standard indirect immunofluorescence assay with serial dilutions of the purified IgG before and after ErbB2 neutralization. After extensive washing, samples were labeled with FITC-conjugated goat anti-mouse Ig, and the cytofluorometric analysis was performed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Saturating amounts of anti-ErbB2 immune IgG were used in all experimental settings.

Adoptive transfer of Abs

One-half milligram of IgG purified from control or immune sera was injected i.p. in BALB-pfpKO recipient mice the day before TUBO challenge and 3, 10, and 17 d after.

Cell stimulation and lysis

TUBO cells (5×10^6 /ml) were left untreated or were stimulated with 100 ng/ml HRG- β 3 for 10 min at 37°C. In some experiments, cells were also incubated for 30 min at 37°C with IgG purified from control or immune sera before and after ErbB2 neutralization. Cells were then lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 mM NaF, 10% glycerol, 1 mM Na₃VO₄, and protease inhibitors. Protein concentration was determined using the Bradford protein assay (Bio-Rad, Hercules, CA), and the normalized samples were used as whole-cell lysates or for immunoprecipitation.

For in vivo studies, tumors of similar size were harvested from BALB- μ IgKO and BALB-pfpKO mice challenged with TUBO cells and vaccinated as above described. Total cell lysates were obtained by resuspending samples in 1% SDS-containing buffer upon repeated freezing and thawing cycles.

Immunoprecipitation and immunoblotting were performed as previously described (39).

PI3K activity assay

TUBO cells (30×10^6 /sample) were treated and lysed in 0.5% Triton X-100 as described above, and equal amounts of total proteins were immunoprecipitated with anti-ErbB3 Ab for 2 h at 4°C. The immunoprecipitates were washed twice with lysis buffer, twice with 0.1 M Tris-HCl (pH 7.5) and 0.5 M LiCl, and twice with 10 mM HEPES (pH 7.3) and 150 mM NaCl. The enzymatic reaction was performed for 25 min at 25°C in 60 μ l kinase buffer (10 mM HEPES [pH 7.3], 1 mM EDTA, 10 mM MgCl₂, 80 μ M ATP, and 0.4 mM adenosine) containing 0.2 μ g/ μ l phosphatidylinositol (sonicated for 15 min at 4°C) and 20 μ Ci [³²P] γ -ATP (6000 Ci/mmol; Amersham Biosciences, Zurich, Switzerland), and it was stopped by adding 1 N HCl. Phosphatidylinositol was extracted with chloroform/methanol (1:1 v/v) and then separated by TLC using chloroform/methanol/ammonium hydroxide/water (86:76:10:14 v/v). The spot corresponding to phosphatidylinositol phosphate was visualized by autoradiography and quantified by densitometric analysis.

PTEN phosphatase assay

TUBO cell lysates, left untreated or treated as above, were immunoprecipitated with anti-PTEN Ab for 2 h at 4°C. After immunoprecipitation, the beads were washed once in lysis buffer; five times in low-stringency buffer containing 20 mM HEPES (pH 7.3), 50 mM NaCl, 0.1 mM EDTA, and 2.5 mM MgCl₂; and once in phosphatase buffer (100 mM Tris-HCl [pH 8] and 10 mM DTT) lacking PIP₃. Assay was performed for 40 min at 37°C in 60 μ l phosphatase buffer containing 100 μ M water-soluble PIP₃ (Echelon Biosciences, Salt Lake City, UT). Release of phosphate from substrate was measured using BIOMOL Green Reagent (BIOMOL, Plymouth Meeting, PA).

Cell growth assay

TUBO cells (2.5×10^4 /well) were resuspended in DMEM supplemented with 1% complemented FBS, seeded in triplicate in a flat-bottom 96-well plate, and exposed to serial dilutions of control or immune IgG (100 μ l/well). Cell growth was indirectly evaluated 96 h posttreatment by mitochondria metabolic activity using MTT assay. TUBO cells were washed twice with RPMI 1640 without phenol red and incubated with 100 μ l 0.5 μ g/ μ l MTT reagent for 3 h at 37°C before 100 μ l stop reagent (0.04 N isopropanol/HCl) was added; the absorbance was measured at 540 nm.

Flow cytometric analysis of cell cycle and apoptosis

TUBO cells were seeded in duplicate at 5×10^5 cells/well in a six-well plate and exposed to 30 or 60 μ g control or immune IgG for 72 h.

For cell cycle analysis, cells were harvested with trypsin-EDTA, washed with cold PBS, and fixed in 70% ethanol for 1 h at 4°C. The cells were stained with 50 µg/ml propidium iodide (PI) supplemented with 250 µg/ml RNase A for 30 min at RT in the dark. DNA content was measured using a FACSCalibur (BD Biosciences).

For the detection of apoptosis, cells were harvested, washed with Binding Buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂), and resuspended in FITC-conjugated Annexin V (Bender MedSystems, Vienna, Austria). After 15 min of incubation at RT, PI was added, and the percentage of Annexin V-FITC and Annexin V-FITC/PI⁺ cells was determined by flow cytometry.

Statistical analysis

Differences in tumor latency were evaluated by the Mann-Whitney *U* test, differences in tumor protection were evaluated by a contingency test (χ^2 test), and differences in apoptosis and cell cycle were evaluated by the Student *t* test. Densitometric analysis of the films was performed using NIH-Image J software (National Institutes of Health, Bethesda, MD).

Results

Protective immunity against ErbB2⁺ tumors involves B cells

TUBO cell challenges are rejected by BALB/c mice electroporated with EC-TM^{neu} plasmids before tumor challenge. In these mice, DNA vaccination elicits anti-rat ErbB2 Abs and a T cell-dependent cytotoxicity (40). To evaluate whether EC-TM^{neu} vaccination induces a protective response on established tumors, BALB/c mice bearing 2-mm-mean diameter TUBO carcinomas (~10 d after TUBO cell challenge) were electroporated with EC-TM^{neu} or control pcDNA3 plasmids. Tumor masses grew progressively in all control mice, giving rise to 10-mm-mean diameter tumors around

day 50 (Fig. 1A, upper panel). In contrast, in mice immunized with EC-TM^{neu} plasmid, tumor masses continued to grow, reaching 4-mm mean diameter; subsequently, tumors were rejected by 80% of mice (5/6; $p < 0.0001$).

To assess the relative importance of the immune mechanisms involved, a similar experiment was performed in BALB- μ IgKO mice that are unable to produce Abs and in BALB-pfpKO mice devoid of perforin-mediated killing mechanisms.

In these immune-deficient mice, the TUBO cells grow faster than in wild-type immunocompetent mice, suggesting a role for Ig- and perforin-mediated responses in tumor surveillance. The fact that the loss of perforin causes an increase in tumor growth suggests that there is a constitutive attack occurring on TUBO cells. Because previous data suggested that CD8⁺ T cells are not activated by a TUBO cell challenge (31, 32), the mechanisms triggered by perforin-containing lymphocytes of innate immunity seem to be primarily involved. Once these mechanisms are removed by the deletion of perforin, tumor control by Ab alone becomes difficult.

None of the BALB- μ IgKO and BALB-pfpKO mice was able to reject the tumor following EC-TM^{neu} plasmid electroporation, indicating that Ig- and perforin-mediated immune responses are required for tumor eradication (Fig. 1A, middle and lower panels). Although the faster growth of TUBO cells following perforin loss makes the tumor much more difficult to control, the EC-TM^{neu} plasmid vaccination was still able to significantly delay the tumor growth in BALB-pfpKO mice, accounting for an important role of Abs in the induction of the protective response.

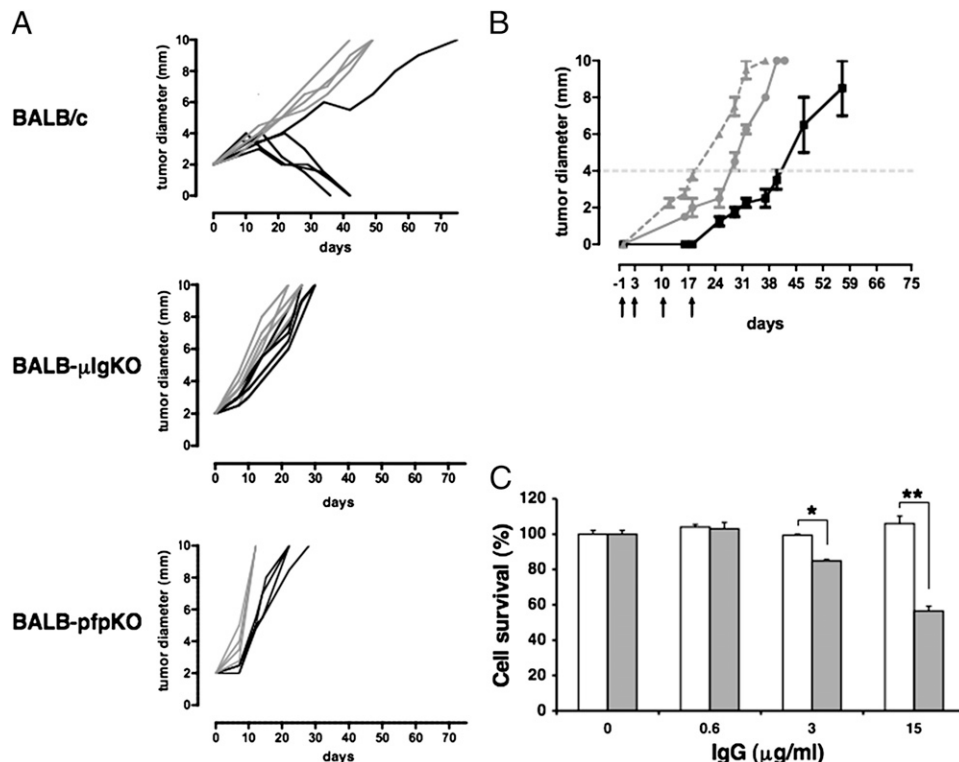


FIGURE 1. A, In vivo mechanisms of TUBO eradication. Tumor growth and regression were evaluated in BALB/c, BALB- μ IgKO, and BALB-pfpKO mice bearing 2-mm-mean diameter carcinomas vaccinated with EC-TM^{neu} (black line) or pcDNA3 plasmid (gray line). EC-TM^{neu} vaccination cured 80% of BALB/c mice (5 of 6; $\chi^2 < 0.0001$) and induced a significant delay in tumor growth in BALB-pfpKO mice (time required for a tumor to go from 2 to 10 mm in mean diameter; $p = 0.023$) but not in BALB- μ IgKO mice. B and C, In vivo and in vitro inhibition of tumor cell growth and survival by anti-ErbB2 Abs. B, Repeated adoptive transfers (arrows) of control (gray line) and immune (black line) IgG in BALB-pfpKO mice. TUBO-injected but nonimmunized BALB-pfpKO mice were included as a negative control (dashed line). C, TUBO cells (2.5×10^5 /well) were exposed to serial dilutions of control (white bars) or immune (gray bars) IgG. The dose of 15 µg/ml of immune IgG corresponded to the saturating amount of vaccine-induced anti-ErbB2 Abs. Cell growth was evaluated after 96 h using the MTT assay. The value of 100% was assigned to untreated samples. Data are expressed as the mean percentage (\pm SD) of three independent experiments. * $p < 0.05$; ** $p < 0.001$.

Vaccine-induced Abs bind rat ErbB2 and inhibit tumor cell growth

The presence of specific anti-rat ErbB2 Abs in a pool of purified IgG from sera of BALB/c mice immunized with EC-TM^{neu} vector (immune IgG) was evaluated in a neutralization experiment. Immune IgG were incubated with an excess of a recombinant rat ErbB2 protein, and its ability to bind TUBO cells or 3T3/NKB fibroblasts stably transfected with the rat ErbB2 was evaluated by flow cytometry (Fig. 2). Total purified IgG from sera of BALB/c mice immunized with empty vector (control IgG) was used as the negative control. Specific binding was observed only when immune IgG was used before ErbB2 neutralization, demonstrating the presence of Abs against rat ErbB2 protein in this pool. The incubation of anti-mouse MHC class I H-2K^d molecule with an excess of the recombinant rat ErbB2 protein did not affect the binding to MHC class I molecule H-2K^d (data not shown).

To evaluate the ability of immune IgG to specifically bind to rErbB2 protein, we performed ELISA assay using a commercially available anti-rat ErbB2 mouse Ab (Ab-4) as the relative standard. We found that a significant fraction (~40–50%) of immune IgG is able to bind to the rErbB2 protein (data not shown).

The contribution of perforin-dependent cytotoxic mechanisms driven by Abs on the growth of TUBO cells in vivo was assessed in BALB-pfpKO mice that received repeated adoptive transfers of immune or control IgG starting 1 d before TUBO cell challenge (Fig. 1B). Although the transferred immune IgG was unable to cure mice, it blocked the tumor growth for the first 2 wk and significantly extended the time required by the tumor to reach 4-mm mean diameter ($p < 0.0285$).

When TUBO cells were cultured for 96 h in the presence of different concentrations of control or immune IgG, a dose-dependent inhibition of cell survival was evident: a reduction of ~50% was reached in the presence of 15 $\mu\text{g/ml}$ immune IgG compared with the same amount of control IgG (Fig. 1C).

Altogether, these results show that vaccine-induced anti-ErbB2 Abs interfere with the expansion of ErbB2⁺ mammary tumor cells by acting directly on tumor cells.

Vaccine-induced anti-ErbB2 Abs impair TUBO cell cycle progression and induce apoptosis

The inhibition of tumor cell growth could be a direct consequence of the inhibition of cell cycle progression and/or of apoptosis induction. To investigate this point, TUBO cells were left untreated or

were incubated with control or immune IgG (Fig. 3). Flow cytometric assessment of cell cycle by means of PI staining revealed a decrease ($33\% \pm 4\%$) in the percentage of cells in the S and G₂/M phases, which correlated with a G₀/G₁ phase increase ($20\% \pm 2\%$) upon treatment with immune IgG (Fig. 3A). In addition, Annexin V/PI staining revealed an increase of up to 3-fold in Annexin V⁺ cells within the immune IgG-treated population compared with control IgG-treated cells (Fig. 3B). We are aware that our experimental approaches cannot exclude that the increased percentage of cells in the G₀/G₁ phase also conceal the incoming apoptosis.

Altogether, our results show that treatment with vaccine-induced anti-ErbB2 Abs inhibits TUBO cell growth by impairing cell cycle progression and inducing apoptosis.

Rat ErbB2-mediated tumorigenic signal in TUBO cells

To elucidate the mechanism(s) responsible for Ab-mediated antitumor activity, we first evaluated the expression of ErbB family members and their physical and functional interactions in TUBO cells (Fig. 4). TUBO cells expressed no detectable levels of ErbB1, elevated levels of ErbB3, a truncated form of ErbB4, and a low level of autochthonous mouse ErbB2 (Fig. 4A). The expression of high levels of the transgenic activated rat *ErbB2* oncogene was also detected, confirming previous evidence based on flow cytometry (31).

Activated rat ErbB2 exhibits a single-point mutation in the TM domain, leading to ligand-independent receptor homo- and heterodimerization (31, 41). Thus, it is likely that on TUBO cells, physical and functional interactions between autochthonous mouse ErbB3 and transgenic rat ErbB2 could occur, despite their cross-species molecular structure.

To test this hypothesis, TUBO cells were left untreated or were stimulated with HRG, one of the natural ErbB3 ligands, and the presence of ErbB2–ErbB3 heterodimers was evaluated. ErbB2/3 complexes (Fig. 4B), as well as ErbB3 (Fig. 4C) and rat ErbB2 (data not shown) tyrosine phosphorylation, were detected in unstimulated cells. HRG stimulation upregulated ErbB3 tyrosine phosphorylation and slightly increased the association between the two receptors. Thus, transgenic ErbB2 and autochthonous ErbB3 are constitutively associated and heavily tyrosine phosphorylated on TUBO cells.

Among the ErbB family dimers, the ErbB2/ErbB3 heterodimer is the strongest activator of the PI3K pathway (10, 16), because

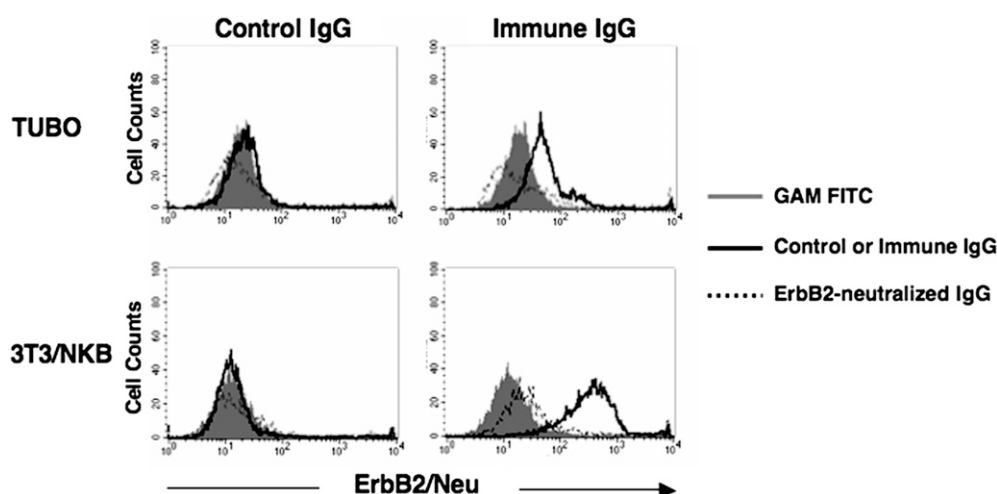


FIGURE 2. Evaluation of vaccine-induced anti-ErbB2 Abs in a pool of affinity-purified IgG. TUBO and 3T3/NKB cells (5×10^5) were stained with control or immune IgG before and after ErbB2 neutralization. The ability to bind to ErbB2 was evaluated by flow cytometry.

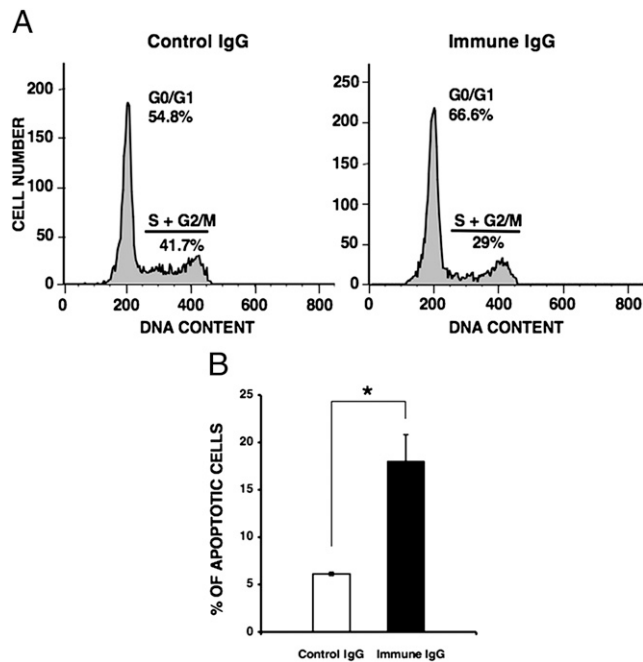


FIGURE 3. Vaccine-induced anti-ErbB2 Abs inhibit TUBO cell cycle progression and induce apoptosis. *A*, TUBO cells (5×10^5) were exposed to 30 μ g of control or immune IgG for 72 h at 37°C. After treatment, cell cycle analysis was performed by flow cytometry. The percentage of TUBO cells in the G₀/G₁ and S+G₂/M phases are shown as DNA graphs. The results represent one of three independent experiments ($n = 3$; G₀/G₁; $p < 0.05$; S+G₂/M; $p < 0.01$). *B*, TUBO cells (5×10^5) were exposed to 60 μ g of control or immune IgG for 72 h at 37°C. The rate of TUBO cell apoptosis was detected by flow cytometry and is represented as the percentage of Annexin V plus Annexin V/PI⁺ cells. Data are expressed as the mean percentage (\pm SD) of three independent experiments. * $p < 0.05$.

ErbB3 displays multiple binding sites for the p85 regulatory subunit of PI3K (14, 15). Consistent with this finding, we found that p85 coprecipitated with ErbB3 in unstimulated and HRG-stimulated TUBO cells (Fig. 4C).

To assess PI3K activation status, we examined Akt phosphorylation in TUBO cells (Fig. 4D). Akt was heavily phosphorylated, and a further increase in its phosphorylation status was observed following HRG stimulation.

Altogether, our results show that TUBO cells coexpress high levels of tyrosine phosphorylated transgenic ErbB2 and autochthonous ErbB3 receptors as homo- and heterodimers and exhibit a basal level of Akt phosphorylation, demonstrating a constitutive activation of the PI3K/Akt pathway.

Anti-ErbB2 Ab-mediated perturbation of Akt phosphorylation status

To investigate whether vaccine-induced anti-rat ErbB2 Abs would perturb PI3K/Akt signaling on TUBO cells, we compared Akt phosphorylation status in cells left unstimulated or stimulated with HRG before and after treatment with control or immune IgG (Fig. 5A, 5B). The basal level of Akt phosphorylation was markedly reduced upon immune IgG treatment with respect to untreated or control IgG-treated cells, whereas no differences in the level of HRG-induced Akt phosphorylation were detected (Fig. 5A). Notably, the impairment in the basal level of Akt phosphorylation observed upon immune IgG treatment was specifically induced by anti-rat ErbB2 Abs, because a lack of perturbation of Akt phosphorylation was observed in the presence of immune IgG upon anti-ErbB2 neutralization (Fig. 5B).

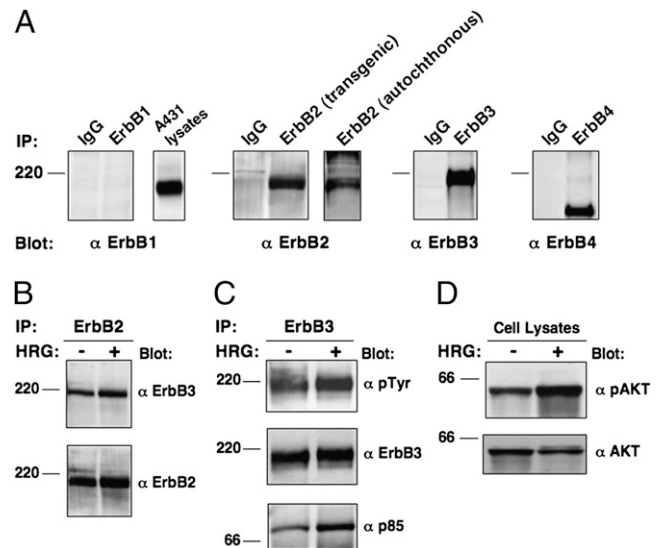


FIGURE 4. ErbB family member interactions in TUBO cells. *A*, TUBO cell lysates (20×10^6 /sample) were immunoprecipitated and immunoblotted with the indicated Abs. A431 cell lysates were used as the positive control. *B* and *C*, TUBO cells left unstimulated (–) or stimulated (+) with 100 ng/ml HRG for 10 min at 37°C were lysed in 0.5% Triton X-100, and cell lysates were immunoprecipitated with anti-ErbB2 (*B*) or ErbB3 (*C*) Abs. The coprecipitated proteins and total cell lysates (*D*) were immunoblotted with the indicated Abs. The results represent one of three independent experiments.

To investigate in vivo whether a vaccination-induced Ab response was able to perturb the PI3K/Akt pathway, we harvested TUBO carcinomas of similar size grown in BALB-pfpKO and BALB- μ IgKO mice electroporated with EC-TM^{neu} plasmid and we evaluated the phosphorylation state of Akt on total cell lysates (Fig. 5C). Higher levels of Akt phosphorylation were observed in tumors from mice unable to produce Abs compared to those from BALB-pfpKO mice.

Taken together, our results show an anti-ErbB2 Ab-mediated perturbation of Akt phosphorylation status in vitro and in vivo.

The impairment of Akt phosphorylation depends on an alteration in PTEN, but not in PI3K, enzymatic activities

We first examined whether immune IgG treatment induced Akt dephosphorylation by inhibiting the enzymatic activity of PI3K. As shown in Fig. 6A by in vitro PI3K assay performed on ErbB3 immunoprecipitates, the constitutive level of ErbB3-associated PI3K enzymatic activity was unaffected by immune IgG treatment, suggesting that the impairment of Akt phosphorylation does not depend on a decrease in PI3K activity. An aliquot of whole-cell lysate was used to assess Akt phosphorylation status (Fig. 6B).

The major negative regulator of the tumorigenic PI3K/Akt signaling pathway is the lipid phosphatase PTEN, which, by dephosphorylating PIP₃, affects Akt membrane recruitment (12, 13). Thus, we asked whether PTEN activation could account for the immune IgG-mediated Akt dephosphorylation.

We assayed the phosphatase activity of PTEN after immunoprecipitation of equivalent amounts of PTEN from untreated, control IgG-treated, or immune IgG-treated TUBO cells (Fig. 6C). Compared with a constitutive low level of PTEN activity in untreated and control IgG-treated cells, 30 min of immune IgG treatment greatly increased PTEN phosphatase activity.

The tyrosine phosphorylation of PTEN indirectly reduces PTEN activation by impairing enzyme localization to the cell membrane (42). Thus, we investigated whether immune IgG treatment would

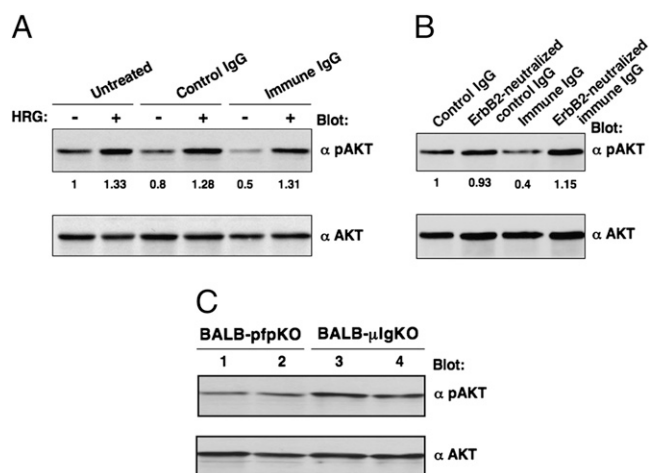


FIGURE 5. Anti-ErbB2 Abs impair Akt phosphorylation in vitro and in vivo. **A**, TUBO cells treated with control or immune IgG for 30 min at 37°C were left unstimulated (–) or were stimulated (+) with 100 ng/ml HRG for 10 min at 37°C. **B**, TUBO cells were treated and stimulated as in **A** before or after anti-ErbB2 Ab neutralization. Total cell lysates were immunoblotted with anti-phospho-Akt (pAkt) and anti-Akt Abs. The numbers indicate the relative amount of pAkt, normalized to the band intensity of total Akt, compared with untreated (**A**) or control IgG-treated (**B**) samples. The results represent one of three independent experiments. **C**, Four tumors of similar size (1–4) growing in two distinct BALB-pfpKO (1 and 2) and BALB-μIgKO (3 and 4) mice were harvested and lysed. Equal amount of proteins, determined by Bio-Rad DC Protein Assay, were immunoblotted with anti-pAkt and anti-Akt mAbs.

affect PTEN tyrosine phosphorylation (Fig. 6D). We found that PTEN is tyrosine phosphorylated in untreated and control IgG-treated TUBO cells; however, the level of PTEN tyrosine phosphorylation was markedly reduced upon immune IgG treatment.

Taken together, our results show that vaccine-induced anti-ErbB2 Abs induce a significant increase in PTEN phosphatase activity that correlates with a reduction in PTEN tyrosine phosphorylation in TUBO cells.

Discussion

ErbB2 functions as an oncogenic unit in a substantial fraction of human cancers (5–7). Because of its oncogenic potential and accessibility, it has emerged as an attractive target for therapeutic strategies based on the use of specific mAbs. In contrast, DNA vaccination against the EC and TM domains of the rat ErbB2 (EC-TM^{neu} plasmid) inhibits the growth of transplanted rat ErbB2⁺ tumors (31, 32) and the onset of autochthonous multifocal carcinomas in rat *ErbB2* transgenic mice (38). Especially in the latter model, tumor halting is mainly due to vaccine-elicited long-term persisting polyclonal anti-rat ErbB2 Abs.

The role of anti-ErbB2 Abs is endorsed by the present data showing the inefficacy of the EC-TM^{neu} vaccine in protecting BALB-μIgKO mice, which are unable to produce Abs, against ErbB2⁺ challenging cells. Conversely, the efficacy of the vaccine and of the adoptive transfer of immune IgG in BALB-pfpKO mice suggests that a significant part of the ability of anti-ErbB2 Abs to delay ErbB2⁺ tumor growth is independent of perforin-mediated cytotoxic mechanisms. In vitro data show that vaccine-elicited anti-rat ErbB2 Abs directly inhibit TUBO cell growth by impairing cell cycle progression and by inducing apoptosis.

Altogether, our results demonstrate that anti-ErbB2 Abs significantly contribute to confer protection by directly interfering with ErbB2 activity.

Activation of the PI3K/Akt pathway is a common event in a large fraction of human cancers and is mainly responsible for the

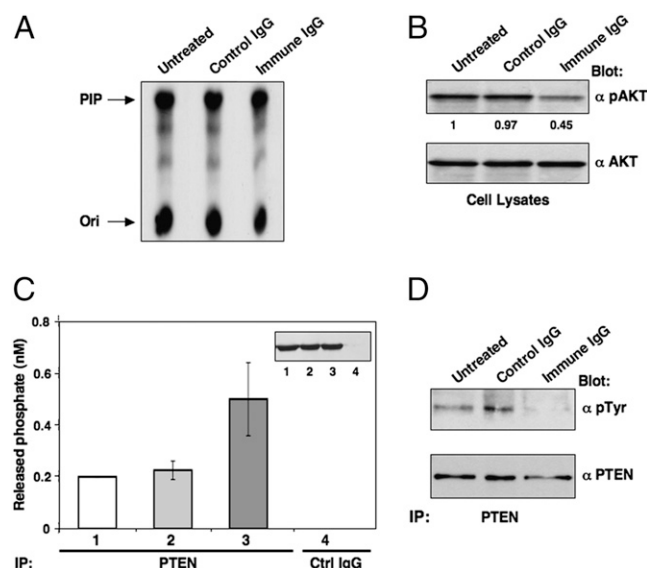


FIGURE 6. Vaccine-induced anti-ErbB2 Abs increase PTEN phosphatase activity without affecting PI3K activity. TUBO cells (30×10^6 /sample) were left untreated or were treated with control or immune IgG. **A**, Cell lysates were immunoprecipitated with anti-ErbB3 mAb and tested in a PI3K assay. The spot corresponding to phosphatidylinositol phosphate was visualized by autoradiography. **B**, Equivalent amounts of cell lysates were used to show the effect of immune IgG on Akt phosphorylation. The numbers indicate the relative amount of pAkt, normalized to the band intensity of total Akt, compared with the untreated sample. **C**, Cell lysates were immunoprecipitated with anti-PTEN (1–3) or control isotype IgG (4) Abs, and the immunoprecipitates from untreated (1 and 4), control (2), or immune IgG-treated (3) TUBO cells were tested for PTEN phosphatase activity. The release of phosphate from substrate was measured using BIOMOL Green Reagent. The inset shows that equal amounts of PTEN were immunoprecipitated from each sample (1–3). Data are expressed as the mean released phosphate (\pm SD) from three independent experiments. **D**, PTEN immunoprecipitates from untreated, control, or immune IgG-treated TUBO cells were immunoblotted with anti-phosphotyrosine and anti-PTEN Abs. The results represent one of three independent experiments.

transformed phenotype (43). In breast cancers, the PI3K/Akt pathway is activated by different mechanisms, including ErbB2 amplification, PI3K mutation, and PTEN inactivation (43–47). Because TUBO cells coexpress tyrosine phosphorylated rat ErbB2 and mouse ErbB3 receptors able to form spontaneous heterodimers, the high basal level of Akt phosphorylation that we observed points to a constitutive activation of the PI3K/Akt pathway.

We further show that the pool of anti-ErbB2 pAbs purified from the sera of vaccinated mice cooperates to significantly reduce the basal level of Akt phosphorylation. The perturbation of PI3K/Akt signaling correlates with TUBO cell growth inhibition observed upon Ab treatment and is consistent with previous findings demonstrating the ability of different anti-ErbB2 mAbs, including herceptin, to inhibit PI3K and Akt activities and tumor cell growth (48, 49).

Accordingly, experiments performed on total cell lysates of harvested tumors growing in vaccinated BALB-pfpKO and BALB-μIgKO mice showed higher levels of Akt phosphorylation in tumors from mice unable to produce Abs compared to those from BALB-pfpKO mice.

The inhibitory effect of anti-ErbB2 Abs was overcome by stimulation with HRG, one of the natural ligands of ErbB3. A likely explanation for this result is that the Abs, although able to perturb the constitutive ErbB2-mediated PI3K/Akt signaling pathway (perhaps disrupting the spontaneous ErbB2 homodimers and/or ErbB2/ErbB3 heterodimers), are unable to interfere with the

stronger coupling of the HRG-activated ErbB2/ErbB3 heterodimers to the PI3K/Akt pathway. This latter possibility is supported by our data showing an increase in p85/ErbB3 complexes upon HRG stimulation in TUBO cells. Our results are consistent with previous findings demonstrating that, in breast cancer cells overexpressing ErbB2, the concomitant presence of ErbB3 confers drug resistance by enabling autocrine or paracrine ErbB3 ligands to promote a full activation of the PI3K/Akt signaling pathway (10, 16).

The perturbation of the basal level of Akt phosphorylation induced by anti-ErbB2 Abs might depend on the inhibition of PI3K activity and/or on the activation of PTEN phosphatase activity. Anti-ErbB2 Abs do not impair the constitutive high level of PI3K enzymatic activity observed on TUBO cells; however, they induce a significant increase in PTEN phosphatase activity that correlates with the inhibition of PTEN tyrosine phosphorylation, which is known to reduce enzyme capacity to bind to the cellular membrane (42). Thus, it is likely that anti-ErbB2 Abs, by reducing PTEN tyrosine phosphorylation, promote the translocation of PTEN from the cytoplasm to the membrane, positively affecting its enzymatic activity. Although we are further investigating this latter possibility, our data are in accord with those of Nagata et al. (42), who demonstrated that PTEN activation contributes to trastuzumab antitumor activity.

In summary, by addressing the ability of vaccine-induced anti-ErbB2 pAbs to inhibit cell growth, our study proposes the perturbation of the PI3K/Akt signaling pathway through the activation of the phosphatase PTEN as a major mechanism of Ab action, even if it is conceivable that optimal *in vivo* activity of a mixture of anti-ErbB2 mAbs involves direct and indirect mechanisms, including Fc-mediated effector functions (28). Furthermore, we previously reported that sera from immunized BALB/c mice efficiently promoted ErbB2R internalization (31, 33). The two modalities of direct Ab action are not mutually exclusive and may depend on the recognition of different ErbB2 epitopes. In this regard, previous studies raised the possibility that combining two or more anti-ErbB2 Abs would increase the therapeutic potential (24–30). Moreover, an immunization protocol aimed at generating persistent pAb production by the patient's own immune system may provide a long-lasting therapeutic benefit, without the need for repeated and expensive Ab administration. In particular, ErbB2-based DNA vaccine holds promise for the treatment of HER2-amplified breast cancer expressing high levels of activated HER3 (50). However, the optimization of such a therapy must take into account the entire system of potentially interacting receptors, as well as tissue environmental conditions, such as the presence of ErbB-specific growth factors.

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Disclosures

The authors have no financial conflicts of interest.

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