

Immunosurveillance of *ErbB2* Carcinogenesis in Transgenic Mice Is Concealed by a Dominant Regulatory T-Cell Self-Tolerance

Elena Ambrosino,¹ Michela Spadaro,¹ Manuela Iezzi,² Claudia Curcio,¹ Guido Forni,³ Piero Musiani,² Wei-Zen Wei,⁴ and Federica Cavallo¹

¹Department of Clinical and Biological Sciences, University of Turin, Orbassano, Italy; ²CeSI, Aging Research Center, "G. D'Annunzio" University Foundation, Chieti, Italy; ³Molecular Biotechnology Center, University of Turin, Torino, Italy; and ⁴Karmanos Cancer Institute, Wayne State University, Detroit, Michigan

Abstract

To assess the role of CD4⁺CD25⁺Foxp3⁺ regulatory T (Treg) cells in overcoming immunosurveillance of *ErbB2* (*HER-2/neu*) mammary lesions, we studied the effects of their sustained removal in BALB/c female mice made transgenic for the rat *ErbB2* (*r-ErbB2*) oncogene (BALB-neuT mice), which develop multiple mammary carcinomas. During the progression of these lesions, Treg cells expand in the spleen, tumor draining lymph nodes, and tumors. Repeated administration of anti-CD25 antibodies extends tumor-free survival, reduces carcinoma multiplicity, and leads to the manifestation of a natural antibody and CTL-mediated reactivity against *r-ErbB2*. Loss of Foxp3⁺ Treg cells during anti-CD25 treatment remarkably caused the disappearance of Gr1⁺ immature myeloid cells, suggesting a cross-talk between these two inhibitory immune cell types. Treg cell expansion associated with *r-ErbB2* overexpression may be seen as a physiologic response to dampen the immune reaction elicited by local anomalous overexpression of a self-antigen. (Cancer Res 2006; 66(15): 7734-40)

Introduction

Overexpression of the *ErbB2* (*HER-2/neu*) oncogene by a significant percentage of human carcinomas is associated with aggressive tumor growth, greater invasiveness, enhanced metastatic potential, and increased resistance to therapy (1). The protein product coded by *ErbB2*, p185, is a member of the epidermal growth factor receptor family endowed with a potent tyrosine kinase activity that plays crucial roles in physiologic processes, such as embryogenesis, cell proliferation, and apoptosis (2). However, as p185 is a self-antigen poorly expressed by the cells of adult individuals, patients with clinically evident carcinomas overexpressing *ErbB2* may display a natural antibody and cell-mediated response to p185 (3, 4). These are too small and too late to enhance resistance to tumor expansion. Their induction, however, suggests that p185 overexpression triggers an immune response. This is an important issue, because elicitation of an immune response to p185 (5) and administration of high-avidity monoclonal antibodies (mAb; ref. 6) are effective against *ErbB2* carcinomas.

The inability to naturally mount an effective immune response to p185 is the result of immune tolerance to self-antigens. Clonal deletion of T and B cells recognizing p185 with high avidity irreversibly impairs the immune repertoire. Lower-affinity responses and responses to subdominant epitopes of self p185 that were not deleted may be inhibited by regulatory T (Treg) cells (7), interleukin (IL)-13-producing natural killer (NK) T cells (8), and immature myeloid cells (9) as well as additional mechanisms providing a negative regulation of the immune response of autoreactive T cells.

To study the surmounting of natural immunosurveillance during *ErbB2* carcinogenesis, we used female BALB/c mice made transgenic for the rat *ErbB2* (*r-ErbB2*) transforming oncogene (BALB-neuT). All these female mice develop a multifocal carcinoma in each of their 10 mammary glands (10) with a stepwise progression that mimics a few typical features of human *ErbB2* carcinogenesis (11). This progression can be exploited to study the natural expansion of CD4⁺CD25⁺ Treg cells expressing the Foxp3 transcription factor and the glucocorticoid-inducible tumor necrosis factor receptor (GITR; ref. 12).

Transgenic *ErbB2* is the only genetic difference between BALB-neuT and wild-type BALB/c mice. They can thus be compared with assess the consequences of progressive overexpression of a self-antigen in the expansion of Treg cells, because these are physiologically involved in inhibiting the immune response and maintaining homeostatic tolerance to self-antigens (12, 13). As Treg cell removal with antibodies or defects in their maturation may result in various forms of autoimmunity (12–15), reduction of their expansion may disclose the presence of a natural immunosurveillance to overexpressed rat p185 (*r-p185*) in BALB-neuT mice and may pose the basis for the design of more effective immunologic maneuvers in tumor prevention and treatment.

The results of the present study show that Treg cells expand in BALB-neuT mice during the lengthy progression of *ErbB2*-driven mammary carcinogenesis. Their sustained removal discloses an antibody and CTL-mediated natural immunosurveillance able to hamper the progression of autochthonous *ErbB2* lesions.

Materials and Methods

Mice. Severe combined immunodeficient (SCID) and BALB/c (H-2^d; 6–8 weeks old) female mice were obtained from Charles River Italia SpA (Calco, Italy). Mammary cancer-prone BALB-neuT female mice (H-2^d) overexpressing the *r-ErbB2* transforming oncogene under the control of the mouse mammary tumor virus promoter (10) were bred for us under specific pathogen-free conditions at Charles River Italia. These mice were randomly assigned to control and treatment groups and concurrently treated. Mammary glands were inspected weekly to note tumor appearance. Progressively growing masses >1 mm mean diameter were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of

Note: Present address for E. Ambrosino: Molecular Immunogenetics and Vaccine Research Section, Vaccine Branch, National Cancer Institute, NIH, Bethesda, MD 20892.

E. Ambrosino and M. Spadaro contributed equally to this work.

Requests for reprints: Federica Cavallo, Department of Clinical and Biological Sciences, University of Turin, Ospedale San Luigi Gonzaga, I-10043 Orbassano, Italy. Phone: 39-11-790-5419; Fax: 39-11-236-5417; E-mail: federica.cavallo@unito.it.

©2006 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-06-1432

incident tumors divided by total number of mice and is reported as mean \pm SE (10). Each neoplastic mass was measured with calipers in two perpendicular diameters and its volume was calculated as $(X^2 \times Y) / 2$, where X and Y represent the short and long diameters, respectively. Total tumor volume is the sum of individual tumor volumes of each mouse and is reported as mean \pm SD. In all operations, mice were treated in accordance with the European Community guidelines.

Production and administration of anti-CD25 antibodies. The PC61 hybridoma-secreting IgG1 mAbs to the α -chain of murine IL-2 receptor (CD25; ref. 16) was purchased from the American Type Culture Collection and cultured *in vitro* in DMEM (BioWhittaker Europe, Verviers, Belgium) supplemented with 5% fetal bovine serum, 0.5 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acid, 1.25 g/L bicarbonate, and 25.7 mmol/L β -mercaptoethanol and then grown as ascites in SCID mice (15). The titer of IgG1 in the ascite fluids passed through 0.45 μ m membrane filters (BD Biosciences, Erembodegem, Belgium) was determined with radial immunodiffusion kits (The Binding Site Ltd., Birmingham, United Kingdom). The fluid was diluted in PBS to obtain a concentration of 2.5 mg IgG1/mL. At the sixth week of age, BALB-neuT mice twice received 500 μ g anti-CD25 IgG or control normal rat IgG (rlgG; Sigma-Aldrich, St. Louis, MO) followed by weekly i.p. repeats until the 24th week.

Morphologic analyses. Histologic evaluation of mammary carcinogenesis and whole mounts of mammary glands were done as described previously in detail on groups of five BALB-neuT mice of progressive age (11, 12). To evaluate the presence of Foxp3⁺ cells in mammary glands, spleen, and lymph nodes during tumor progression and Treg cell depletion, groups of five BALB-neuT mice untreated or receiving normal rlgG or anti-CD25 IgG were sacrificed at ages 7, 13, 19, and 25 weeks. For immunohistochemistry, pyridoxal phosphate-fixed tissues were embedded in OCT and acetone-fixed cryostat sections were incubated for 60 minutes with anti-Foxp3 (clone MF333F, Alexis Italia, Vinci, Florence, Italy). Microwave antigen retrieval was done with 1 mol/L urea for 3 minutes. After washing, sections were overlaid with biotinylated goat anti-rlgG (Vector Laboratories, Burlingame, CA) for 30 minutes, incubated with streptavidin ABC/alkaline phosphatase. Staining was developed with fuxin (DakoCytomation, Heverlee, Belgium). For each mouse, Foxp3⁺ lymphoid cells were counted in a blind fashion independently by three pathologists in 10 high-power \times 400 fields.

Cytometric identification of Treg cells and CD11b⁺Gr1⁺ immature myeloid cells. The relative numbers of CD4⁺CD25⁺Foxp3⁺GITR⁺ Treg and CD11b⁺Gr1⁺ immature myeloid cells in the spleen and lymph nodes draining the mammary pad were evaluated by flow cytometry. Spleen cells (Spc; 1×10^6) and cells from lymph nodes draining the mammary pad were treated with Fc receptor blocker (CD16/CD32; PharMingen, San Diego, CA) for 15 minutes at 4°C. For Treg cell detection, directly conjugated phycoerythrin (PE) anti-mouse GITR (clone DTA-1, eBioscience, San Diego, CA), PE/Cy7 anti-mouse CD4 (clone GK1.5, BioLegend, San Diego, CA), and allophycocyanin anti-mouse CD25 (clone PC61.5, eBioscience) were incubated for 30 minutes at 4°C. The cells were then washed in PBS with 0.1% sodium azide and 2% fetal bovine serum. Cell pellets were resuspended in 1 mL Fix/Perm (eBioscience) and the samples were incubated overnight at 4°C. After two washes with permeabilization buffer (eBioscience), the samples were incubated with 2 μ L Fc receptor blocker for 15 minutes at 4°C and then with FITC anti-mouse/rat Foxp3 (FJK-16s, eBioscience) for 30 minutes at 4°C and washed twice before analysis. For immature myeloid cell detection, cells were incubated with 1 μ L PE anti-mouse Ly-6G and Ly-6C (Gr-1, clone RB6-8C5, PharMingen) diluted 1:20 in PBS and 3 μ L FITC anti-mouse CD11b (Mac1 α , clone M1/70, PharMingen) at 4°C for 30 minutes. The samples were washed twice with PBS containing 0.1% sodium azide and 2% calf serum and analyzed on the CyAn ADP (DakoCytomation) through Summit 4.2 (DakoCytomation) software.

Assessment of anti-r-p185 antibody. Sera collected at 10 and 25 weeks from BALB-neuT mice receiving normal rlgG or anti-CD25 IgG were diluted 1:50 in PBS/sodium azide/bovine serum albumin (Sigma-Aldrich) and the presence of anti-r-p185 antibodies was determined by flow cytometry using BALB/c NIH3T3 fibroblasts, wild-type or stably cotransfected with the wild-type *r-ErbB2*, mouse class I H-2K^d and B7.1 genes (BALB/c NIH3T3-NK B

cells; ref. 17). FITC-conjugated goat antibodies specific for mouse IgG Fc (DakoCytomation) were used to detect bound primary antibodies. Normal mouse serum was the negative control. The mAb Ab4 (Oncogene Research Products, Cambridge, MA), which recognizes an extracellular domain of r-p185, was used as a positive control. Serial Ab4 dilutions in normal mouse serum were used to generate a standard curve to determine the concentration (μ g/mL) of anti-r-p185 antibodies in mouse sera. Flow cytometry was done on the CyAn ADP (17).

In vitro and in vivo CTL assays. Spc were restimulated *in vitro* by culturing for 6 days 1×10^7 cells with 5×10^5 mitomycin C (Sigma-Aldrich)-treated 3T3NKb cells in the presence of 10 units/mL recombinant IL-2 (Eurocetus, Milan, Italy). In some experiments, Spc were restimulated by adding 2.5 μ g/mL of the r-p185 63-71 nonamer peptide (TYVPANASL; ref. 18; InBios Srl, Biotech Products, Naples, Italy) predicted to bind the H-2^d glycoproteins with high affinity (<http://www.syfpeithi.de/>). Restimulated Spc were assayed in a 4-hour Na₂⁵¹CrO₄ (⁵¹Cr, Perkin-Elmer, Boston, MA) release assay at E:T ratios from 50:1 to 6:1 in round-bottomed, 96-well microtiter plates in triplicate as described previously in detail (19). *In vivo* cytotoxicity assay was done as described by Ritchie et al. (20), with slight modifications. Briefly, a single-cell suspension of 10^7 naive Spc/mL was labeled with 0.5 or 5.0 μ mol/L of the fluorescent dye CFSE (Molecular Probes, Leiden, the Netherlands). Spc labeled with 5 μ mol/L CFSE (CFSE^{high}) were also pulsed with r-p185 63-71 nonamer peptide for 1 hour at room temperature. The two Spc populations were mixed together in equal amounts and injected i.v. into control and treated mice. Mice were sacrificed 48 hours later, and single-cell suspensions from spleens were processed individually to evaluate the presence of CFSE^{high} and CFSE^{low} cells with the CyAn ADP after adding propidium iodide to exclude dead cells. The specific cytolytic activity was calculated as $100 \times (\text{percentage CFSE}^{\text{low}} \text{ cells} - \text{percentage CFSE}^{\text{high}} \text{ cells}) / \text{percentage CFSE}^{\text{low}} \text{ cells}$.

Statistics. Differences in tumor incidence were evaluated with the Mantel-Haenszel log-rank test, those in tumor multiplicity, number of positive cells at flow cytometry and antibody titer with Student's two-tailed *t* test.

Results

Treg cells expand during *ErbB2* carcinogenesis. First, we determined whether the progression of carcinogenesis in the mammary glands of BALB-neuT mice triggers the expansion of CD4⁺CD25⁺Foxp3⁺ Treg cells. The atypical mammary hyperplasia generated by cells overexpressing r-p185 first evident at age 4 weeks (11) progresses to multifocal preneoplastic lesions around week 7 (Fig. 1C and H). Multiple carcinomas of $\sim 200 \text{ mm}^3$ are palpable in every mouse by week 25 (Fig. 1A, F, and K). At the seventh week, the initial *ErbB2* overexpression does not lead to detectable Treg cell accumulation in the spleen (Fig. 1A) and the axillary lymph nodes draining the mammary pad (data not shown) or in the mammary lesions, where Foxp3⁺ lymphoid cells remain 1 ± 2 per $\times 400$ microscopic field (Fig. 2A). However, the subsequent progression of mammary carcinogenesis is accompanied by an increment in CD4⁺CD25⁺Foxp3⁺ Treg cells in the spleen (Fig. 1A) and the mammary tumors (Fig. 2B). We have shown previously that this progression of BALB-neuT carcinogenesis is also accompanied by an expansion of CD11b⁺Gr1⁺ immature myeloid cells (21).

Sustained depletion of CD4⁺CD25⁺Foxp3⁺GITR⁺ Treg cells through chronic infusion of anti-CD25 IgG. Cancer-prone BALB-neuT mice received repeated infusions of normal rlgG or anti-CD25 IgG from the 6th week to the 24th week of age to assess their effect on Treg cells. A dramatic decrease in Treg cells was already evident at week 7 in both the spleen (Fig. 3A) and axillary lymph nodes (Fig. 3B) following the first two administrations of 500 μ g anti-CD25 IgG. At week 25, the percentage of Foxp3⁺GITR⁺ T cells among total CD4⁺ cells almost doubled in the spleen and

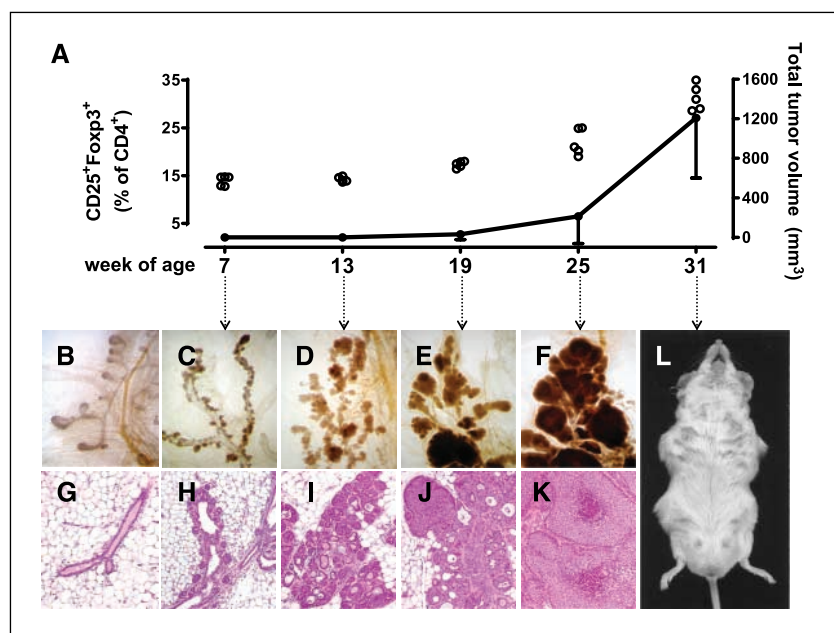


Figure 1. Kinetics of tumor progression and CD4⁺CD25⁺Foxp3⁺ Treg cell expansion. A, percentage of CD25⁺Foxp3⁺ cells among total CD4⁺ Spc (left axis, dots) and total tumor volume (right axis, line) evaluated at 6-week intervals in untreated cancer-prone BALB-neuT mice. At each time point, the percentage of CD25⁺Foxp3⁺ cells was evaluated individually in groups of 5 mice. There were 25 mice in the experiment at the beginning, and for each determination of CD25⁺Foxp3⁺ cells, 5 mice were sacrificed after total volume evaluation. Total tumor volume is the mean \pm SD of 25-5 mice. Whole mounts (B-F) and histologic sections (G-K) of mammary glands from BALB/c and BALB-neuT mice. B and G, saccular terminal end buds (B) and normal duct lined by a single layer of ductal epithelial cells (G) of normal 7-week-old BALB/c mice. In BALB-neuT mice age 7 weeks (C and H), ducts are associated with little dark side buds (C) that histologically appear as ductal protrusions (H). At week 13, these structures surround the ducts (D) and give rise to lobules of atypical hyperplasia and foci of *in situ* carcinoma (I) that expand and converge into lobular carcinoma (E and J). At week 25, large masses (F) of carcinoma with a nodular appearance and divided by solid septa often display central necrosis (K). At week 31, macroscopic tumors are evident in all the 10 mammary glands (L). Magnification, $\times 20$ (B and C), $\times 16$ (D-F), and $\times 200$ (G-K). Representative images from groups of five mice whose each mammary gland was examined as described in Materials and Methods.

markedly increased in the lymph nodes of normal rIgG-treated BALB-neuT mice. By contrast, repeated injections of anti-CD25 IgG, twice in week 6 and then once weekly until week 24, keeps low their percentage in both spleen and lymph node as shown by flow cytometry (Fig. 3A and B). This reduction is mirrored by immunocytochemistry observations (Fig. 3C versus Fig. 3D).

Treg cell depletion accompanies delayed carcinogenesis. To determine whether chronic removal of Treg cells unveils an immune response able to hamper the progression of *ErbB2* lesions, tumor incidences were compared in normal rIgG-treated and anti-CD25 IgG-treated BALB-neuT mice. Both a marked delay in the appearance of the first tumor and a reduction in the number of palpable tumors per mouse (tumor multiplicity; ref. 11) were evident in anti-CD25 IgG-treated mice. At week 20, when all control mice displayed one or more tumors, 80% of mice treated with anti-CD25 IgG were free of palpable tumors (Fig. 4A). The tumor-free survival curve of anti-CD25 IgG-treated mice was significantly delayed ($P < 0.0001$) compared with that of normal rIgG-treated mice. The tumor multiplicity was also significantly lower ($P < 0.04$ to $P < 0.0004$) from weeks 19 to 32 (Fig. 4B). Moreover, at 38 weeks, when the experiment ended, a few mammary glands of BALB-neuT mice receiving anti-CD25 IgG until week 24 did not display a palpable tumor. Depletion of Treg cells through anti-CD25 IgG also leads to both a reduced number of CD11b⁺Gr1⁺ immature myeloid cells at week 7 and their drastically hindered expansion at week 25 (Fig. 5).

Treg cell depletion unveils a natural immune response to r-p185. We next determined whether Treg cell expansion conceals an immune response that may be naturally triggered by r-p185 overexpression. A single weekly infusion of anti-CD25 IgG, but not normal rIgG, uncovers both a significant antibody (Fig. 6) and a CTL response to r-p185 (Fig. 7). The presence of anti-r-p185 natural antibodies was assayed in the sera from 10- and 25-week-old BALB-neuT mice repeatedly infused with normal rIgG or anti-CD25 IgG. A significant higher titer of anti-r-p185 antibodies was found in sera from anti-CD25-treated mice at both age 10 weeks ($P = 0.0045$) and age 25 weeks ($P = 0.028$) compared with age-matched normal rIgG-treated control mice (Fig. 6). No anti-r-p185 antibodies were

detectable in untreated 10- and 25-week-old BALB/c mice (data not shown). The CTL response of Spc from anti-CD25-treated 25-week-old BALB-neuT mice was studied against both target cells pulsed with the r-p185 63-71 dominant H-2K^d restriction element peptide (Fig. 7A and B) and target cells expressing the whole r-p185 (Fig. 7C). The *in vivo* cytotoxic response against r-p185 63-71 peptide-pulsed cells (Fig. 7A) was significantly higher in anti-CD25-treated mice compared with normal rIgG-treated mice ($P < 0.0001$). The cytotoxic response of Spc from anti-CD25-treated BALB-neuT mice was confirmed *in vitro* against cells pulsed with the r-p185 63-71 peptide (Fig. 7B; Spc from anti-CD25 versus normal rIgG-treated mice, 48.5 ± 0.6 versus 14.2 ± 0.4 LU₂₀/10⁷ cells; $P < 0.0001$) and against 3T3NKB cells expressing r-p185 (Fig. 7C; Spc from anti-CD25 versus normal rIgG-treated mice, 45.1 ± 0.5 versus 6.3 ± 0.3 LU₂₀/10⁷ cells; $P < 0.0001$). No cytotoxicity against r-p185 was detectable in Spc from untreated 10- and 25-week-old BALB/c mice (data not shown). The presence of a marked cell-mediated cytotoxic response to r-p185 is impressive because it was never observed in tolerant BALB-neuT mice even after repeated anti-r-p185 immunizations (19, 22-25).

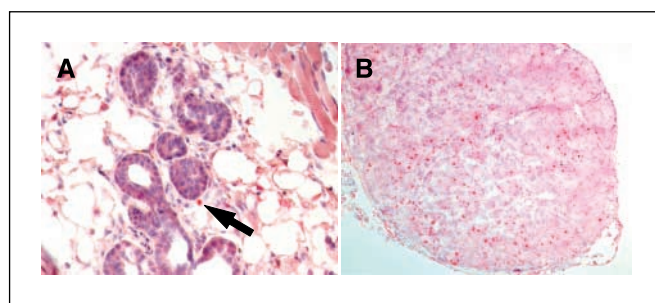


Figure 2. Accumulation of Foxp3⁺ cells in the mammary lesions. Immunohistochemical staining of Foxp3⁺ cells (red) on mammary tissue obtained from BALB-neuT mice at week 7 (A) and week 25 (B). A, a single positive cell (arrow) is close to a hyperplastic mammary duct ($\times 400$); B, several positive cells are infiltrating the solid tumor mass ($\times 100$). Representative images from groups of five mice whose each mammary gland was examined as described in Materials and Methods.

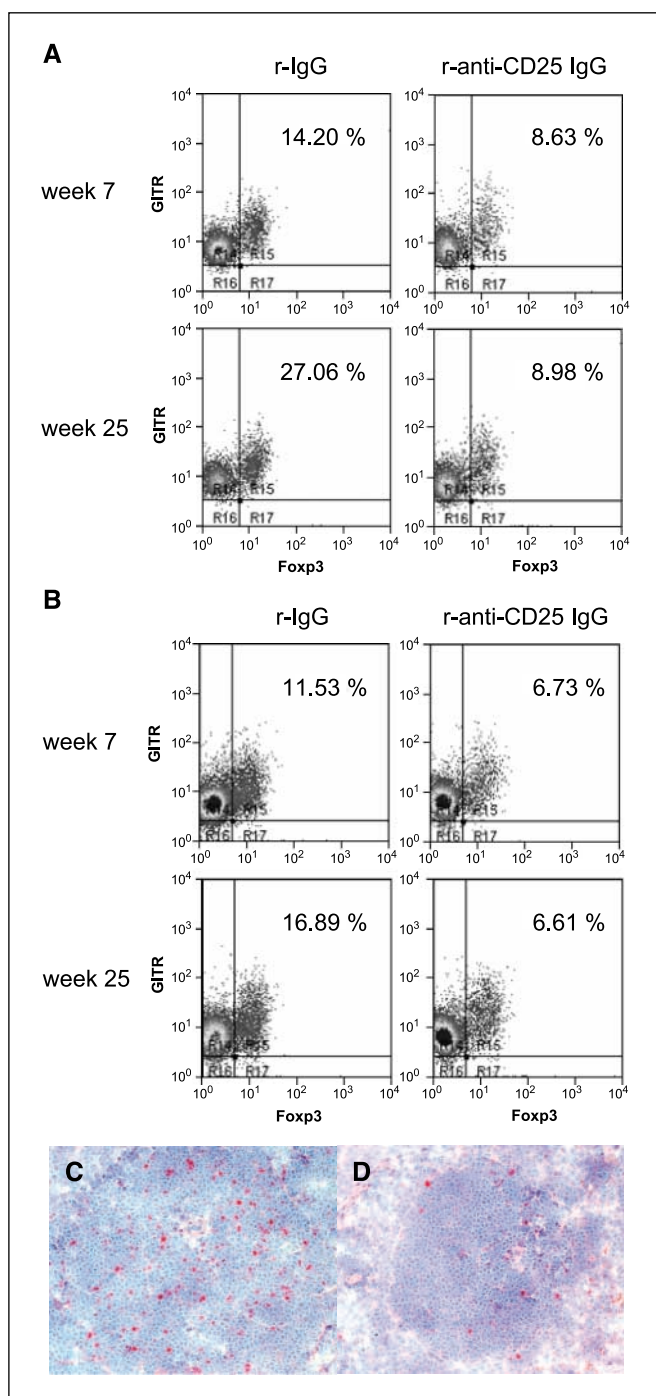


Figure 3. Foxp3⁺GITR⁺ Treg cells are depleted by anti-CD25 treatment. Following two administrations of 500 μ g anti-CD25 IgG (week 6), the percentage of Foxp3⁺GITR⁺ T cells markedly drops in both the spleen (A) and the axillary lymph nodes (B) of BALB-neuT mice. At week 25, although Foxp3⁺GITR⁺ T cells increase during mammary carcinogenesis in BALB-neuT treated with normal rIgG, the repeated injections of anti-CD25 IgG, twice in week 6 and then once weekly until week 24, keep low the percentage of Foxp3⁺GITR⁺ T cells. Positively stained CD4⁺ T cells were gated and Foxp3 and GITR profiles were depicted as dot plots. The number in the top right quadrant represents the percentage of Foxp3⁺GITR⁺ cells within the CD4⁺ T cell population. Representative dot plot from one of the five mice individually analyzed and that gave homogeneous results. C and D, immunohistochemical staining of Foxp3⁺ cells (red) in the spleens from 25-week-old BALB-neuT mice. The numerous Foxp3⁺ cells present in the T-cell-rich periarteriolar area of the spleens from BALB-neuT mice treated with normal rIgG (C) are drastically reduced by repeated weekly injections of anti-CD25 IgG (D). Representative images from groups of five mice examined as described in Materials and Methods.

Discussion

Enhancement of immunosurveillance through Treg cell removal has been mostly reported in transplantable tumor models (26–30) but also with tumors growing in transgenic mice (17, 30) and chemically induced tumor development (31). Here, we show that during *ErbB2*-driven mammary carcinogenesis CD4⁺CD25⁺Foxp3⁺GITR⁺ Treg cells expand in the spleen, tumor draining lymph nodes, and mammary lesions. The progression of BALB-neuT carcinogenesis is also accompanied by an expansion of CD11b⁺Gr1⁺ immature myeloid cells in both the blood (21) and the spleen. Chronic infusion of anti-CD25 IgG into BALB-neuT mice does not simply result in the inactivation of CD4⁺CD25⁺ cells (32) but also leads to a sustained physical depletion of CD4⁺CD25⁺Foxp3⁺GITR⁺ Treg cells. Such removal unveils a natural immunosurveillance against *ErbB2*-driven autochthonous carcinogenesis.

Because of the mammary overexpression of membrane r-p185, transgenic BALB-neuT mice are genetically predestined to develop multiple invasive and metastasizing mammary carcinomas (10). Many features of their progression, including gene expression profiles, closely mimic what happens in human mammary cancer (11, 33). In these mice, the chronic removal of CD4⁺CD25⁺Foxp3⁺GITR⁺ Treg cells extends tumor-free survival, reduces carcinoma multiplicity, and leads to the manifestation of a natural antibody and CTL-mediated reactivity against r-p185. It also hinders the expansion of CD11b⁺Gr1⁺ immature myeloid cells that goes along with tumor progression (21).

Because the r-*ErbB2* transgene is the genetic difference between wild-type BALB/c mice and transgenic BALB-neuT mice, comparison of the immune response in these two lines allows direct assessment of the tolerance to r-p185 as an overexpressed

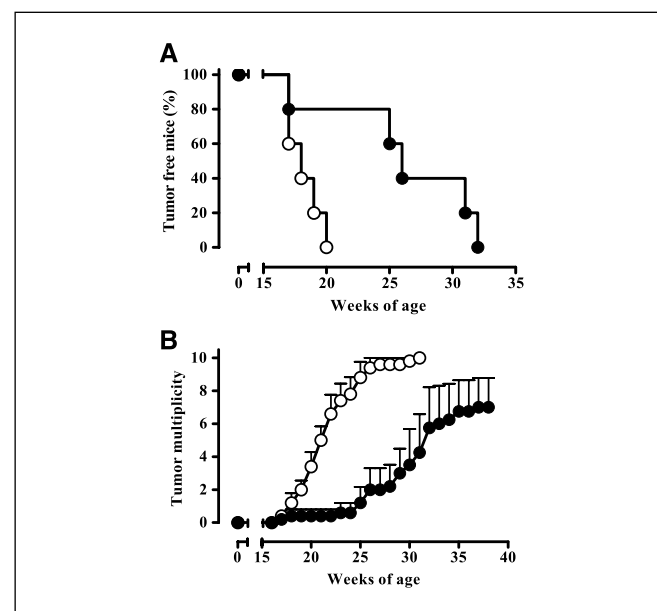


Figure 4. Anti-CD25 treatment delays mammary carcinogenesis. Tumor-free survival (A) and tumor multiplicity (B) in BALB-neuT mice receiving infusions of 500 μ g anti-CD25 IgG (●) or normal rIgG (○) twice in week 6 and then once weekly until week 24. A, tumor incidence in anti-CD25 IgG-treated mice is significantly different ($P < 0.0001$) compared with that of normal rIgG-treated mice. B, weeks when tumor multiplicity in anti-CD25 IgG is significantly different from that of normal rIgG-treated mice: weeks 19 and 20 ($P < 0.04$), weeks 21 and 22 ($P < 0.001$), weeks 23 to 28 ($P < 0.0009$), week 29 ($P < 0.003$), and weeks 30 to 32 ($P < 0.03$).

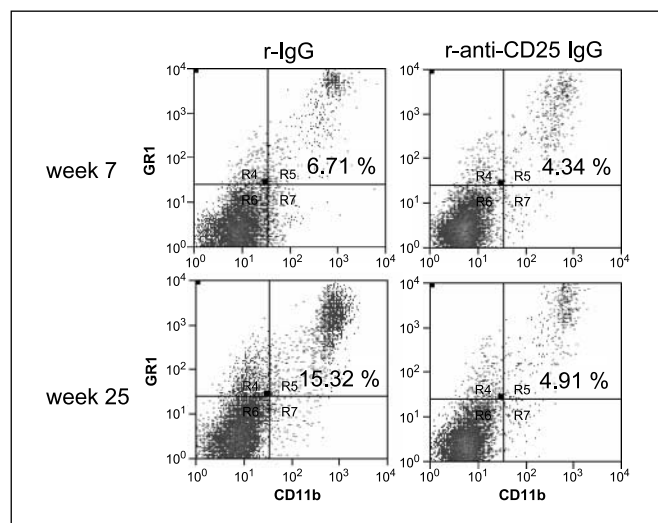


Figure 5. Depletion of Treg cells hampers the expansion of CD11b⁺Gr1⁺ immature myeloid cells. In the spleen of BALB-neuT mice treated with 500 μ g anti-CD25 IgG twice in week 6 and then once weekly until week 24, Treg depletion goes along with CD11b⁺Gr1⁺ immature myeloid cell reduction at week 7 (*top right*) and with a lack of their expansion that accompanies mammary carcinogenesis (21) at week 25 (*bottom right*). GR1 and CD11b profiles of total Spc were depicted as dot plots. The number in the top right quadrant represents the percentage of CD11b⁺Gr1⁺ cells within the Spc. Representative dot plot from one of the five mice individually analyzed and that gave homogeneous results.

tumor-associated antigen. BALB/c mice do not express r-p185, which is thus a xenogeneic antigen differing in several epitopes from mouse p185 (18). Following immunization, BALB/c mice develop a strong immune response to r-p185, and CTL are a significant component of such response (34). The reaction triggered by the vaccine (34) or after Treg cell removal (15) is strong enough to bring about the rejection of large transplanted r-p185⁺ tumors. By contrast, in BALB-neuT mice, r-p185 is expressed in the thymus at birth and is progressively increasingly overexpressed by the cells of hyperplastic mammary lesions starting from the fourth week of age (11). Because this r-p185 overexpression, immunoscope analysis of the T-cell repertoire shows that in BALB-neuT mice CTL clones reacting with high affinity with r-p185 peptides are depleted.⁵ CD4 T-cell clones able to recognize r-p185 peptides are still present and vaccines elicit an IFN- γ - and antibody-mediated immune response that hampers the initial stages of autochthonous carcinogenesis, whereas the CTL response is not evident (19, 22–25, 35).

Very little information is available regarding how tumor-specific Treg cells develop in tumor-bearing hosts. Present data in BALB-neuT mice show that by comparison with age-matched BALB/c mice no major increase in Treg cells is evident during the early stages of mammary hyperplasia. This is not surprising because r-p185 is but one of the innumerable self-antigens against which the autoimmune response is prevented by Treg cells (36). However, as mammary lesions progress and many more cells overexpress r-p185, an expansion of Treg cells becomes evident in the spleen and particularly in the tumors. This late infiltration of Treg cells in *Erbb2* carcinomas fits in well with what has been described with transplantable tumors (29) and human cancers (37),

where Treg cell accumulation is a late event in well-established tumors. The Treg cell ability to localize to the tumor site seems to permit the close contacts with effector CTL required for interference with their functions. By contrast, Treg cells in the peripheral organs may inhibit CD4 helper function and thus the elicitation of a significant and long-lasting antibody-mediated response (38).

The Treg cell expansion that accompanies r-p185 overexpression may be seen as a physiologic response to dampen the immune reaction elicited by local anomalous overexpression of a self-antigen, a major source of spontaneous autoimmunity (39). However, r-p185 is not only a self-antigen overexpressed on the cell membrane as carcinogenesis progresses but also a signaling receptor that delivers signals triggering the proliferation and survival of normal and tumor cells and whose anomalous overexpression plays a causal role in the promotion of carcinogenesis (1, 2). This double role of r-p185, a self-tolerated antigen playing important physiologic roles and a tumor antigen causally involved in the neoplastic progression, paradigmatically illustrates what may happen with most tumor-associated antigens. These, in fact, are self-antigens and thus display a natural immune recognition and immunosurveillance counterbalanced by a dominant immune tolerance (40, 41).

In several cases, the antigen presented by autochthonous tumors does not promote the dendritic cell activation necessary for proper arousal of effector CD4⁺ and CD8⁺ T-cell responses and results in the induction of tolerance (42, 43). By contrast, present data as well studies in patients (3, 4) suggest that tumor overexpression of p185 is enough to overcome self-tolerance and arouse antibody and cell-mediated immune responses. In the clinical setting, these responses are too small and too late to influence tumor progression. In BALB-neuT mice, they are meaningless as they are buried by Treg cells. Following Treg cell removal, a significant natural surveillance against *Erbb2* carcinogenesis is evident. It is, however, only temporarily effective and insufficient to ultimately eradicate the tumor. This failure may rest on the central deletion in BALB-neuT mice of effector T cells recognizing r-p185 with high

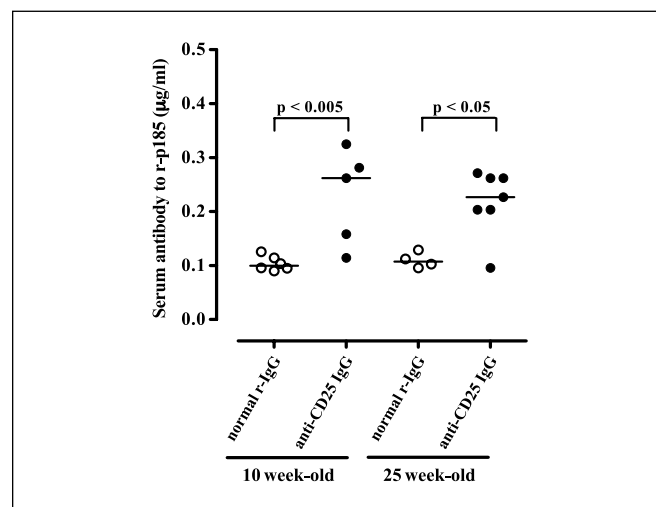
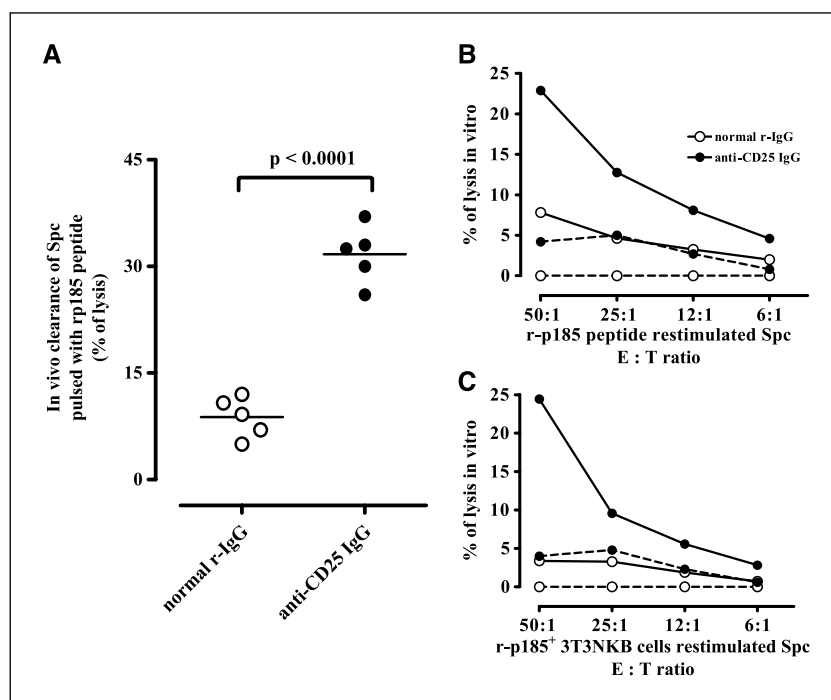


Figure 6. Depletion of Treg cells unveils a natural antibody response to r-p185. Titers of anti-r-p185 antibodies and median (horizontal line) in the sera of BALB-neuT mice repeatedly infused with normal rIgG (○) or anti-CD25 IgG (●). BALB-neuT mice receiving anti-CD25 IgG (five mice at week 10 and seven mice at week 25) displayed a higher titer than mice receiving normal rIgG (six mice at week 10 and four mice at week 25). No anti-r-p185 antibodies were detectable in untreated 10- and 25-week-old BALB/c mice (data not shown).

⁵ Rolla et al., submitted for publication.

Figure 7. Depletion of Treg cells unveils a CTL response to r-p185. Cytotoxicity displayed by 25-week-old BALB-neuT mice infused repeatedly with normal rIgG (○) or anti-CD25 IgG (●). A, mice were injected i.v. with 10×10^6 BALB/c Spc pulsed with r-p185 63-71 peptide and stained with 5 $\mu\text{mol/L}$ CFSE or nonpulsed and stained with 0.5 $\mu\text{mol/L}$ CFSE. Forty-eight hours later, Spc fluorescence was evaluated by flow cytometry and the percentage of lysis was calculated as described in Materials and Methods. B and C, 4-hour ^{51}Cr release assay against r-p185⁺ 3T3NKB cells (continuous lines) or r-p185⁻ BALB 3T3 fibroblasts (dotted lines) with effector Spc restimulated *in vitro* with r-p185 63-71 peptide (B) or r-p185⁺ 3T3NKB cells (C). A to C, five mice in each group; B and C, representative experiment.



avidity. Moreover, additional physiologic immunoregulatory mechanisms (8, 9, 21) can be brought into play by the continuous onset of new neoplastic cells in transgenic BALB-neuT mice (19). Treg and myeloid immature cells are intimately associated with the immune suppression mediated by spontaneous tumors (7, 21). It has been shown that the accumulation of myeloid immature cells driven by tumor expansion of a transplantable tumor favors the expansion of Treg cells (44). On the other hand, present data show that Treg cell depletion avoids the accumulation of myeloid immature cells. Although this may depend on the delayed carcinogenesis due to Treg cell removal, a cross-talk between these two regulatory cells cannot be ruled out, and further studies may elucidate the pathways of their interaction.

In conclusion, present data show that r-p185 overexpression by mammary lesions naturally activates an antibody- and CTL-mediated immunity able to counteract initial stages of carcinogenesis. This, however, is dampened by Treg cells and possibly by other regulatory mechanisms. There are several reasons why Treg cell activity becomes dominant during *ErbB2* carcinogenesis. Overexpression of r-p185 by the BALB-neuT mammary lesions may build the right conditions leading to Treg cell expansion or the conversion of naive CD4⁺ T cells into Treg cells (38). Besides the peculiar cytokines produced by the BALB-neuT carcinomas and their microenvironment, the dominant action of Treg cells may rest on the higher avidity with which they recognize self-antigen

compared with effector T cells that escape deletional tolerance (45). It is evident that the risk of a rampant autoimmunity to an overexpressed self-antigen is a more effective evolutionary pressure than the production of a crippled immunosurveillance. However, the coexistence of dominant regulatory mechanisms and autoimmune-based immunosurveillance is both intriguing and alarming, because maneuvers leading to Treg cell removal may uncover significant antitumor reactivity but also trigger significant autoimmunity to self-antigens (15). In the specific case of *ErbB2*, interference with regulatory mechanisms may improve the effectiveness of immunosurveillance and immunotherapy treatments along with the activation of autoimmune reactions. However, the low avidity of autoimmune effector T cells that escape deletional tolerance (45) will react mostly if not solely against target cells that overexpress p185. In adult life, such overexpression is confined to neoplastic cells.

Acknowledgments

Received 4/19/2006; accepted 5/12/2006.

Grant support: Italian Association for Cancer Research, Italian Ministries for the Universities and Health, University of Torino, Regione Piemonte, and Center of Excellence on Aging, University of Chieti, Italy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Irene Merighi for excellent technical assistance.

References

- Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science* 1987;235:177-82.
- Yarden Y, Slivkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127-37.
- Disis ML, Calenoff E, McLaughlin G, et al. Existent T cell and antibody immunity to HER-2/*neu* protein in patients with breast cancer. *Cancer Res* 1994;54:16-20.
- Disis ML, Knutson KL, Schiffman K, Rinn K, McNeel DG. Pre-existent immunity to the HER-2/*neu* oncogenic protein in patients with HER-2/*neu* overexpressing breast and ovarian cancer. *Breast Cancer Res Treat* 2000;62:245-52.
- Peoples GE, Gurney JM, Hueman MT, et al. Clinical trial results of a HER2/*neu* (E75) vaccine to prevent recurrence in high-risk breast cancer patients. *J Clin Oncol* 2005;23:7536-45.
- Hortobagyi GN. Trastuzumab in the treatment of breast cancer. *N Engl J Med* 2005;353:1734-6.
- Ercolini AM, Ladle BH, Manning EA, et al. Recruitment of latent pools of high-avidity CD8(+) T cells to the antitumor immune response. *J Exp Med* 2005;201:1591-602.
- Terabe M, Matsui S, Noben-Trauth N, et al. NKT

- cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* 2000;1:515–20.
9. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 2005;16:53–65.
 10. 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;188:589–96.
 11. Pannellini T, Forni G, Musiani P. Immunobiology of HER-2/*neu* transgenic mice. *Breast Dis* 2004;20:33–42.
 12. Sakaguchi S. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–52.
 13. Shevach EM. Regulatory T cells in autoimmunity. *Annu Rev Immunol* 2000;18:423–49.
 14. Samy ET, Parker LA, Sharp CP, Tung KS. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4⁺CD25⁺ regulatory T cells in the regional lymph node. *J Exp Med* 2005;202:771–81.
 15. Wei WZ, Jacob JB, Zielinski JF, et al. Concurrent induction of antitumor immunity and autoimmune thyroiditis in CD4⁺CD25⁺ regulatory T cell-depleted mice. *Cancer Res* 2005;65:8471–8.
 16. Moreau JL, Nabholz M, Diamantstein T, Malek T, Shevach E, Theze J. Monoclonal antibodies identify three epitope clusters on the mouse p55 subunit of the interleukin 2 receptor: relationship to the interleukin-2-binding site. *Eur J Immunol* 1987;17:929–35.
 17. Spadaro M, Ambrosino E, Iezzi M, et al. Cure of mammary carcinomas in HER-2 transgenic mice through sequential stimulation of innate (neoadjuvant interleukin-12) and adaptive (DNA vaccine electroporation) immunity. *Clin Cancer Res* 2005;11:1941–52.
 18. Nagata Y, Furugen R, Hiasa A, Ikeda H, Ohta N, Furukawa K. Peptides derived from a wild-type murine proto-oncogene c-erbB-2/HER2/*neu* can induce CTL and tumor suppression in syngeneic hosts. *J Immunol* 1997;159:1336–43.
 19. Quaglino EM, Iezzi C, Mastini C, et al. Electroporated DNA vaccine clears away multifocal mammary carcinomas in HER-2/*neu* transgenic mice. *Cancer Res* 2004;64:2858–64.
 20. Ritchie DS, Hermans IF, Lumsden JM, Scanga CB, Roberts JM, Yang J. Dendritic cell elimination as an assay of cytotoxic T lymphocyte activity *in vivo*. *J Immunol Methods* 2000;246:109–17.
 21. Melani C, Chiodoni C, Forni G, Colombo MP. Myeloid cell expansion elicited by the progression of spontaneous mammary carcinomas in c-erbB-2 transgenic BALB/c mice suppresses immune reactivity. *Blood* 2003;102:2138–45.
 22. Rovero S, Amici A, Di Carlo E, et al. DNA vaccination against rat HER-2/*neu* p185 more effectively inhibits carcinogenesis than transplantable carcinomas in transgenic BALB/c mice. *J Immunol* 2000;165:5133–42.
 23. Nanni P, Nicoletti G, De Giovanni C, et al. Combined allogeneic tumor cell vaccination and systemic interleukin 12 prevents mammary carcinogenesis in HER-2/*neu* transgenic mice. *J Exp Med* 2001;194:1195–205.
 24. Sakai Y, Morrison BJ, Burke JD, et al. Vaccination by genetically modified dendritic cells expressing a truncated *neu* oncogene prevents development of breast cancer in transgenic mice. *Cancer Res* 2004;64:8022–8.
 25. Park JM, Terabe M, Sakai Y, et al. Early role of CD4⁺ Th1 cells and antibodies in HER-2 adenovirus-vaccine protection against autochthonous mammary carcinomas. *J Immunol* 2005;174:4228–36.
 26. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25⁺ CD4⁺ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 1999;163:5211–8.
 27. Golgher D, Jones E, Powrie F, Elliott T, Gallimore A. Depletion of CD25⁺ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur J Immunol* 2002;32:3267–75.
 28. den Boer AT, van Mierlo GJ, Fransen MF, Melief CJ, Offringa R, Toes RE. CD4⁺ T cells are able to promote tumor growth through inhibition of tumor-specific CD8⁺ T-cell responses in tumor-bearing hosts. *Cancer Res* 2005;65:6984–9.
 29. Yu P, Lee Y, Liu W, et al. Intratumor depletion of CD4⁺ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors. *J Exp Med* 2005;201:779–91.
 30. Tien AH, Helgason CD. Altered immunity accompanies disease progression in a mouse model of prostate dysplasia. *Cancer Res* 2005;65:2947–55.
 31. Nishikawa H, Kato T, Tawara I, et al. Accelerated chemically induced tumor development mediated by CD4⁺CD25⁺ regulatory T cells in wild-type hosts. *Proc Natl Acad Sci U S A* 2005;102:9253–7.
 32. Kohm AP, McMahon JS, Podojil JR, et al. Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4⁺CD25⁺ T regulatory cells. *J Immunol* 2006;176:3301–5.
 33. Astolfi A, Rolla S, Nanni P, et al. Immune prevention of mammary carcinogenesis in HER-2/*neu* transgenic mice: a microarray scenario. *Cancer Immunol Immunother* 2005;54:599–610.
 34. Curcio C, Di Carlo E, Clynes R, et al. Nonredundant roles of antibody, cytokines and perforin for the immune eradication of established HER-2/*neu* carcinomas. *J Clin Invest* 2003;111:1161–70.
 35. 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;64:2858–64.
 36. Sakaguchi S. Naturally arising Foxp3-expressing CD25⁺ CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat Immunol* 2005;6:345–52.
 37. Dranoff G. The therapeutic implications of intra-tumoral regulatory T cells. *Clin Cancer Res* 2005;11:8226–9.
 38. Von Boehmer H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* 2005;4:338–44.
 39. Samy ET, Parker LA, Sharp CP, Tung KS. Continuous control of autoimmune disease by antigen-dependent polyclonal CD4⁺CD25⁺ regulatory T cells in the regional lymph node. *J Exp Med* 2005;202:771–81. Erratum in: *J Exp Med* 2005;202:1153.
 40. Houghton AN. Cancer antigens: immune recognition of self and altered self. *J Exp Med* 1994;180:1–4.
 41. Pardoll D. Does the immune system see tumors as foreign self? *Annu Rev Immunol* 2003;21:807–39.
 42. Lyman MA, Aung S, Biggs JA, Sherman LA. A spontaneously arising pancreatic tumor does not promote the differentiation of naive CD8⁺ T lymphocytes into effector CTL. *J Immunol* 2004;172:6558–67.
 43. Willimsky G, Blankenstein T. Sporadic immunogenic tumors avoid destruction by inducing T-cell tolerance. *Nature* 2005;437:141–6.
 44. Huang B, Pan PY, Li Q, et al. Gr-1⁺CD115⁺ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res* 2006;66:1123–31.
 45. Schwartz RH. Natural regulatory T cells and self tolerance. *Nat Immunol* 2005;6:327–30.

Immunosurveillance of *ErbB2* Carcinogenesis in Transgenic Mice Is Concealed by a Dominant Regulatory T-Cell Self-Tolerance

Elena Ambrosino, Michela Spadaro, Manuela Iezzi, et al.

Cancer Res 2006;66:7734-7740.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/66/15/7734>

Cited articles This article cites 43 articles, 27 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/66/15/7734.full#ref-list-1>

Citing articles This article has been cited by 20 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/66/15/7734.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/66/15/7734>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.