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# ErbB2 DNA Vaccine Combined with Regulatory T Cell Deletion Enhances Antibody Response and Reveals Latent Low-Avidity T Cells: Potential and Limits of Its Therapeutic Efficacy

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Rat (r)ErbB2 transgenic BALB-neuT mice genetically predestined to develop multiple invasive carcinomas allow an assessment of the potential of a vaccine against the stages of cancer progression. Because of rErbB2 expression in the thymus and its overexpression in the mammary gland, CD8<sup>+</sup> T cell clones reacting at high avidity with dominant rErbB2 epitopes are deleted in these mice. In BALB-neuT mice with diffuse and invasive in situ lesions and almost palpable carcinomas, a temporary regulatory T cells depletion combined with anti-rErbB2 vaccine markedly enhanced the anti-rErbB2 Ab response and allowed the expansion of latent pools of low-avidity CD8<sup>+</sup> T cells bearing TCRs repertoire reacting with the rErbB2 dominant peptide. This combination of a higher Ab response and activation of a low-avidity cytotoxic response persistently blocked tumor progression at stages in which the vaccine alone was ineffective. However, when diffuse and invasive microscopic cancers become almost palpable, this combination was no longer able to secure a significant extension of mice survival. *The Journal of Immunology*, 2010, 184: 6124–6132.

The consistent stepwise progression of tumors arising in tumor-prone genetically engineered mice allows the assessment of the ability of a vaccine to both provide protection against progressive stages of neoplasia and to elicit a response from an immune system negatively imprinted by the slow expansion of an autochthonous tumor. In these cancer models, vaccination can significantly prevent neoplastic progression for nearly as long as a mouse's natural life span but is usually unable to cure advanced microscopic lesions (1).

This loss of efficacy is due to a tumor's increasing ability to augment its mitotic activity, clonal diversification, and inaccessibility to the mechanisms of the immune reaction. However, the efficacy of

a vaccine administered to mice bearing diffuse microscopic lesions is also diminished by the tumor-driven expansion of various kinds of negative regulatory cells (1). Numerous experiments show that this expansion results in both a far less significant immune response and suppression of its effector arm (2–9).

Tumor-driven regulatory cells expansion also changes the tumor-specific T cell repertoire (5, 10) as a result of both direct contacts and the production of soluble factors such as TGF- $\beta$  or IL-10. In a special way, regulatory T (T<sub>reg</sub>) cells may inhibit the reaction of low-avidity T cells against tumor Ags (10).

This study assesses the significance of T<sub>reg</sub> cell expansion in the inefficacy of vaccination against different stages of tumor progression in female BALB/c mice transgenic for the transforming rat (r) *ErbB2/neu* (rErbB2) oncogene (BALB-neuT mice) (11) that develop invasive and metastatic mammary cancer (12). We have previously shown that expression of the rErbB2 protein product (ErbB2) in their thymus and mammary glands has a dramatic impact on the repertoire of CD8<sup>+</sup> T cells that reach the periphery. By contrast with wild-type BALB/c mice, anti-rErbB2 immunization does not elicit a detectable cytotoxic response in BALB-neuT mice (13, 14). Because they are devoid of the CD8<sup>+</sup> T cell clones that react in the wild-type with high avidity against the immunodominant rErbB2 peptide (15), their antitumor reactivity is mostly confined to CD4<sup>+</sup> T cells and Ab production (15–17).

Hyperplastic foci appear from the 4th wk of age in all mammary glands of BALB-neuT mice, expand to multiple areas of atypical hyperplasia and give rise to disseminated tumor cells and micro-metastasis in bone marrow and lungs (10th wk), and progress to in situ carcinomas (14th wk), which expand and merge to give rise to carcinomas that are initially microscopic (16th wk), then become diffuse and invasive (18th wk) and almost palpable around the 20th wk (12, 18). This progression is accompanied by rampant immune suppression. Expansion of T<sub>reg</sub> cells (19) and immature myeloid cells (20) along with NKT cells (21) progressively inhibits natural

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Abbreviations used in this paper: EC-TM, extracellular and transmembrane domains of the protein product of rErbB2; LN, lymph node; r, rat; RSI, rate of stimulation index; SPC, spleen cell suspension; T<sub>reg</sub>, regulatory T.

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immune surveillance. We have shown that chronic infusion of anti-CD25 Ig results in a sustained depletion of  $T_{reg}$  cells, greatly delays carcinogenesis, and unveils a natural immune response to rErbB2 (19).

In this study, we set out to determine whether anti-rErbB2 DNA vaccination combined with temporary  $T_{reg}$  cell deletion provides better protection against stages of mammary lesions whose progression can no longer be inhibited by vaccination alone. In BALB-neuT mice harboring microscopic *in situ* lesions and almost palpable carcinomas, this combination markedly enhanced the anti-rErbB2 Ab response and allowed both the expansion of latent pools of low-avidity CD8<sup>+</sup> T cells bearing TCR repertoires reacting with the rErbB2 dominant peptide and their activation against this peptide. The result was a persistent blockade of tumor progression at stages in which the vaccine alone was ineffective, whereas survival of mice with diffuse, invasive, and almost palpable microscopic lesions was not significantly extended.

## Materials and Methods

### Mice

SCID mice, wild-type BALB/c mice, and BALB-neuT 6-wk-old female mice were from Charles River Laboratories Italia (Calco, Italy). BALB-neuT mice overexpress the rErbB2 transforming oncogene under the control of the mouse mammary tumor virus promoter and develop a multifocal and metastatic carcinoma in each of their 10 mammary glands with a stepwise progression (12, 18). BALB-neuT mice unable to produce Abs (BALB-neuT/BKO mice) were generated by crossing BALB-neuT mice with BALB/c mice knocked out for the Ig  $\mu$ -chain gene (17). Mice were randomly assigned to control and treatment groups, and all groups were treated concurrently. Mammary glands were inspected weekly to note tumor appearance. Neoplastic masses were then measured with calipers in two perpendicular diameters, and the average value was recorded. Progressively growing masses >1 mm mean diameter were regarded as tumors. Tumor 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. Tumor multiplicity was calculated as the cumulative number of incident tumors/total number of mice at the time of death. Values are reported as mean  $\pm$  SEM. Mammary whole-mount preparations were performed as reported previously (19). According to our ethical protocol, mice were killed when a first tumor exceeded 10 mm mean diameter. All the experiments were approved by the institutional ethical committee, and mice were treated in accordance to the European Union guidelines.

### Production and administration of anti-CD25 Ig

The PC61 hybridoma-secreting IgG1 mAb to the  $\alpha$ -chain of murine IL-2R (CD25) was purchased from the American Type Culture Collection (Manassas, VA) and cultured in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% FBS (Life Technologies, Milan, Italy), 0.5 mmol/l sodium pyruvate, 1 mmol/l nonessential amino acid, 1.25 g/l bicarbonate, and 25.7 mmol/l 2-ME and then grown as ascites in SCID mice. The titer of IgG1 in the ascite fluids passed through 0.45- $\mu$ m membrane filters (BD Biosciences, Erembodegem, Belgium) was determined with radial immunodiffusion kits (The Binding Site, Birmingham, U.K.). The fluid was diluted in PBS (Sigma-Aldrich) to obtain a concentration of 2.5 mg/ml as previously described in detail (19). At the specified times, BALB-neuT mice received 0.5 mg anti-CD25 or rat IgG1 isotype control (eBioscience, San Diego, CA) injected *i.p.*

### Cytometric identification of $T_{reg}$ cells

The relative numbers of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup>  $T_{reg}$  cells in the lymph nodes (LNs) draining the mammary pad were evaluated by flow cytometry. A total of  $1 \times 10^6$  cells were treated with FcR blocker (CD16/CD32; BD Pharmingen, San Diego, CA) for 15 min at 4°C. Directly conjugated PE anti-mouse GITR (clone DTA-1; eBioscience), 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 min at 4°C. The cells were then washed in PBS (Sigma-Aldrich) with 0.1% sodium azide and 2% BSA (PBS-azide-BSA) (Sigma-Aldrich). Cell pellets were suspended 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  $\mu$ l FcR blocker for 15 min at 4°C and then with FITC anti-mouse/rat FoxP3 (FJK-16s; eBioscience) for 30 min at 4°C. The samples were washed twice with PBS-azide-BSA and analyzed on the CyAn ADP (DakoCytomation, Glostrup, Denmark) through Summit 4.2 (DakoCytomation) software.

### Preparation of DNA plasmids and DNA electroporation

The pcDNA3 vector coding the extracellular and transmembrane domains of the rErbB2 (EC-TM plasmid) was produced and used as previously described (22) with slight modifications. Fifty micrograms of EC-TM or empty pcDNA3 plasmid in 20  $\mu$ l sterile water with 0.9% NaCl was injected into the quadriceps muscle of anesthetized mice. Immediately after, two electrical pulses of 375 V/cm of 25 ms in duration were applied using Cliniporator device, and linear needle electrodes (Igea, Carpi, Italy) were inserted in the muscle. Each electroporation course consisted of two plasmid administrations with an interval of 14 d.

### Immunohistochemistry and histology of the mammary glands

For immunohistochemistry, 6- $\mu$ m cryostat sections were air-dried and fixed in ice-cold acetone for 10 min. Slides were incubated with anti-CD8 primary Ab (Abcam, Cambridge, U.K.) and then with the biotinylated secondary Ab. Immunoreactive Ags were detected using NeutrAvidin Alkaline Phosphatase Conjugated (Thermo Scientific-Pierce Chemical Co., Rockford, IL) and Vulcan Fast Red (Biocare Medical, Concord, CA) or Diaminobenzidine Chromogen System (DakoCytomation, Carpinteria, CA). For histological evaluation, tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4  $\mu$ m, and stained with H&E. Tumor vessel were analyzed by immunofluorescence with anti-CD31 (marker of endothelial cells) (BD Pharmingen), anti-NG2 (marker of nascent pericytes) (Chemicon International, Temecula, CA), and anti- $\alpha$ -smooth muscle actin (marker of mature pericytes) (Sigma-Aldrich).

### Ab response

Sera from mice of each treatment group were diluted 1/100 in PBS-azide-BSA. The presence of Abs against rErbB2 was determined by flow cytometry using BALB/c 3T3 fibroblasts wild-type or stably cotransfected with rErbB2 and mouse class I H-2K<sup>d</sup> and B7.1 genes (BALB/c 3T3-NKB) (23). These cells were cultured in DMEM supplemented with 20% FBS at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. FITC-conjugated goat anti-mouse Abs specific for mouse IgG Fc (DakoCytomation) were used to detect bound primary Abs. Normal mouse serum was used as negative control. The mAb Ab4 (Oncogene Research Products, Cambridge, MA), which recognizes an extracellular domain of rErbB2, was used as a positive control. After washing, cells were suspended in PBS-azide-BSA containing 1 mg/ml propidium iodide to exclude dead cells and evaluated in a CyAn ADP (DakoCytomation). The results are expressed as 3T3-NKB-specific binding potential calculated as follows: [(% positive cells with test serum) [fluorescence mean] - (% positive cells with control serum) [fluorescence mean]]  $\times$  serum dilution as previously described in detail (24).

### Cytotoxic T lymphocyte assays

To prepare target cells for *in vivo* cytotoxicity detection (25), erythrocytes in BALB/c spleen cell suspension (SPC) were removed by osmotic lysis. Cells were then washed and split into two target populations. One was labeled with 5  $\mu$ M CFSE (Molecular Probes Invitrogen, Carlsbad, CA), then pulsed with the dominant 63–71 rErbB2 peptide (p63–71, TYVPA-NASL; INBIOS Srl, Naples, Italy) with H-2K<sup>d</sup> restriction element (26) at a concentration of 3 or 15  $\mu$ g/ml for 1 h at 37°C (CFSE<sup>high</sup> cells). The control SPCs were left without peptides and labeled with 0.5  $\mu$ M CFSE (CFSE<sup>low</sup> cells). For injection, an equal number of SPCs from each population were mixed together. Recipient mice were injected *i.v.* with  $20 \times 10^6$  cells suspended in 0.3 ml PBS. Mice were bled 18 h later, and PBMCs were analyzed using flow cytometry as we described previously (19). To detect IFN- $\gamma$  production, lymphocytes from LNs or SPCs were cultured in presence of 3, 15, or 50  $\mu$ g/ml of the p63–71 for 18 h plus 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich) for the last 5 h. Cells were stained with anti-mouse allophycocyanin-CD8 (BD Pharmingen) and then fixed, permeabilized, and stained intracellularly with anti-mouse PE IFN- $\gamma$  (eBioscience).

### T cell repertoire analysis

Repertoire analysis was performed as previously described in detail (15). Popliteal and inguinal LN cells were collected from the various groups of mice, and  $5 \times 10^6$ /ml cells were cultured for 3 d in the presence of 3, 15,

or 50  $\mu\text{g/ml}$  p63–71 or mitomycin C (Sigma-Aldrich)-treated 3T3-NKB cells. Culture medium was RPMI 1640 medium (Life Technologies, Basel, Switzerland) supplemented with 2 mM L-glutamine, 50  $\mu\text{M}$  2-ME, 50  $\mu\text{g/ml}$  gentamicin (all from Sigma-Aldrich), and 10% FBS. Total RNA was isolated from CD4<sup>+</sup> and CD8<sup>+</sup> immunobeads sorted cells (15) with the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. cDNA synthesized using an oligo-dT primer (dT15) (Invitrogen) was subjected to PCR amplification using a common C $\beta$  primer in combination with the various V $\beta$  primers as previously described in detail (15). Using 2  $\mu\text{l}$  PCR product as a template, runoff reactions were performed with the internal fluorescent J $\beta$  primers (15, 27). The runoff products were denatured in formamide and analyzed on an Applied Biosystems 3100 Prism using GeneScan 2.0 software (Applied Biosystems, Foster City, CA). Results are reported as rate of stimulation index (RSI = normalized peak area obtained from cells stimulated with Ag/normalized peak area of nonstimulated cells).

### CD8 depletion

For CD8<sup>+</sup> T cell depletion, BALB-neuT mice were injected with 50  $\mu\text{g}$  anti-mouse CD8 mAb (Cederlane Laboratories, Hornby, Ontario, Canada) every 10 d starting the day before the first vaccination. Three weeks later, mice were challenged s.c. with  $1 \times 10^5$  TUBO cells, an Erbb2<sup>+</sup> cloned cell line established in vitro from a lobular carcinoma that arose in a female BALB-neuT mouse (13).

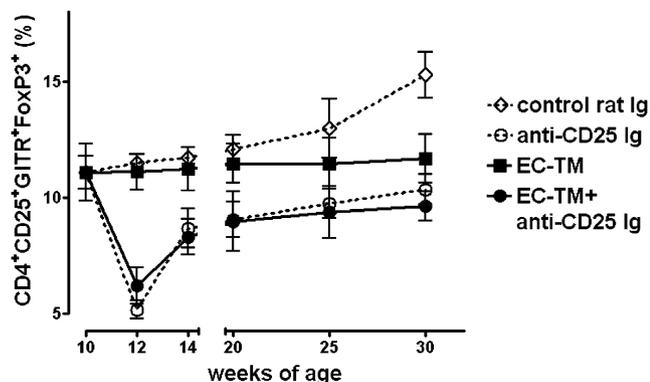
### Statistics

Differences in data were evaluated with the two-tailed Student *t* test, except that differences in tumor incidence were evaluated with the Mantel-Haenszel log-rank test (11).

## Results

### Anti-CD25 Ig persistently impairs T<sub>reg</sub> cell expansion during rErbb2 carcinogenesis

At the 10th week of age, BALB-neuT mice display atypical hyperplasia foci scattered in all 10 mammary glands (18) and disseminated tumor cells and micrometastasis in bone marrow and lungs (12). Progression of these lesions to in situ and invasive cancer is accompanied by the expansion of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells (19). To examine the effect of anti-CD25 Ig on T<sub>reg</sub> cells in tumor draining LNs, 10-wk-old BALB-neuT mice received two injections of control or anti-CD25 Ig 4 d apart. Although a progressive expansion of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup> cells was evident in mice receiving control Ig, 2 wk after the injections of anti-CD25 Ig, their number fell dramatically and remained significantly lower than in control mice until about week 30 (Fig. 1).



**FIGURE 1.** Sustained depletion of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells in tumor draining LN of BALB-neuT mice following anti-CD25 Ig administration. At the 10th week of age, BALB-neuT mice with diffuse preneoplastic lesions in all mammary glands receive an injection of 500  $\mu\text{g}$  anti-CD25 Ig (○) or control rat Ig (◇) on days 2 and 5. Other mice were electroporated with the EC-TM plasmid on the first day of weeks 10 and 12 (■), whereas other mice were electroporated with the EC-TM plasmid and injected with anti-CD25 Ig (●). At each time points, the percentage of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup> cells was evaluated individually in groups of six mice. The mean value and range (vertical bars) are shown.

### Anti-CD25 Ig delays cancer progression and enhances the antitumor protection afforded by EC-TM plasmid electroporation

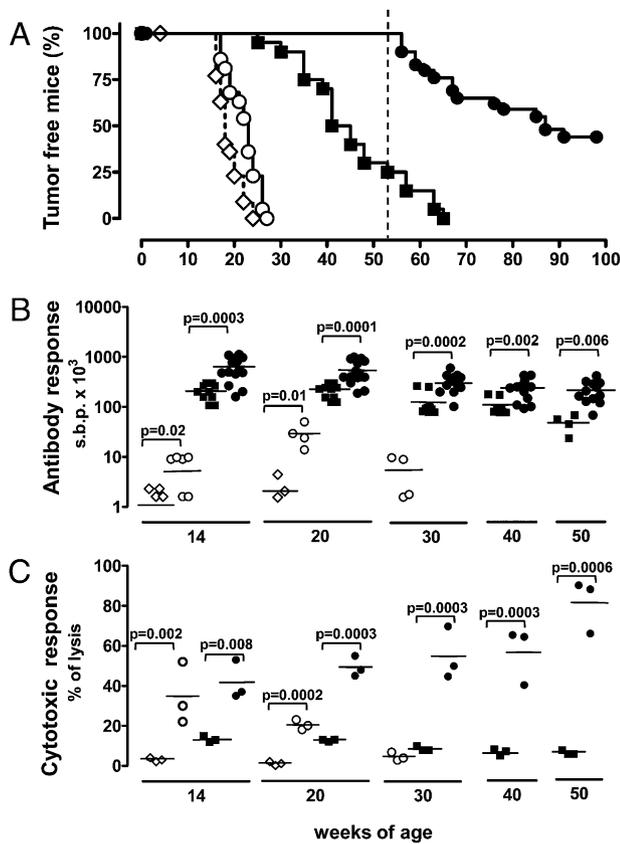
Although the two injections of anti-CD25 Ig alone at week 10 were enough to elicit a small but significant delay in the appearance of mammary tumors, all the treated BALB-neuT mice displayed at least one palpable tumor by week 27 (Fig. 2A). EC-TM plasmid electroporation at weeks 10 and 12 almost doubled the tumor-free survival, but all mice were dead at week 65. When the first electroporation of EC-TM plasmid was combined with anti-CD25 Ig 1 and 4 d later, tumor-free survival exceeded 1 y (Fig. 2A), and 44% of mice were still alive at 100 wk of age, when the experiment ended, and those that developed tumors displayed a lower multiplicity (Supplemental Table I).

### Immune mechanisms enhanced by the combination of anti-CD25 Ig and EC-TM plasmid electroporation

The Ab response to rErbb2 and the cytotoxic response in vivo against the immunodominant H-2K<sup>d</sup> p63–71 (26) were evaluated at progressive times after both the two anti-CD25 Ig injections and EC-TM plasmid electroporation. The two injections at week 10 elicited a small but significant Ab response (Fig. 2B) and a marked cytotoxic response (Fig. 2C). Both responses were evident 4 and 10 wk later and then faded. A significant and persistent Ab response but a marginal cytotoxicity followed EC-TM plasmid electroporation. However, the combination of EC-TM plasmid electroporation and anti-CD25 Ig injection resulted in both a stronger Ab response and a marked and persistent cytotoxic response (Fig. 2B, 2C).

### Anti-CD25 Ig administration unveils CD8<sup>+</sup> T cells participating in tumor halting

A marked cytotoxic response to rErbb2 following anti-CD25 Ig administration, alone or in combination with EC-TM plasmid electroporation, was a new and unexpected finding, because it had never been found in BALB-neuT mice when different protocols of immunizations were adopted (1, 13, 22, 28). Indeed, BALB-neuT mice lack the CD8<sup>+</sup> T cells reacting with high avidity with the rErbb2 immunodominant p63–71 (15). To look for the mechanisms whereby T<sub>reg</sub> cell depletion allows anti-rErbb2 vaccination to elicit such a response, we evaluated the p63–71-specific T cell repertoire thus expanded. Wild-type BALB/c mice and BALB-neuT mice were electroporated at weeks 10 and 12 with EC-TM plasmid. A few of them, together with untreated mice, received anti-CD25 Ig 1 and 4 d after the first electroporation. At week 14, popliteal and inguinal LN cells were collected and cultured with the p63–71. The 288 V $\beta$ -J $\beta$  primer combinations were used to perform the first CDR3 length fragment analysis on a pool of cDNA from five treated BALB-neuT mice. After in vitro restimulation with p63–71, Ag-driven perturbation of normal CDR3- $\beta$  profile was observed in eight spectra from this pool (Fig. 3A). These rearrangements were then checked in other 10 (individual) mice. Three groups of rearrangements specific for the p63–71 were shared among sorted CD8<sup>+</sup> T cells from treated mice: V $\beta$ 1-J $\beta$ 1.1 of 137 bp length (used by 4 of 10 mice), V $\beta$ 6-J $\beta$ 2.7 of 109 or 115 bp length (each used by 6 of 10 mice), and V $\beta$ 7-J $\beta$ 1.2 of 134 or 137 bp length (used by 3 of 10 and 4 of 10 mice, respectively) (Fig. 3B). V $\beta$ 9-J $\beta$ 1.2 rearrangements (98 and 104 bp length) specific for p63–71, used by the vast majority of BALB/c mice electroporated with or without anti-CD25 Ig, were never expanded in any of the BALB-neuT mice (Table I), in keeping with our previous finding (15). These data hint that the p63–71-specific repertoire expanded following T<sub>reg</sub> cell depletion in BALB-neuT mice does not overlap with that activated by EC-TM plasmid electroporation in BALB/c mice, whether alone or in combination



**FIGURE 2.** The combination of EC-TM plasmid electroporation with anti-CD25 Ig administration enhances tumor-free survival and induces a persistent Ab and cytotoxic response. At the 10th week of age, groups of BALB-neuT mice with diffuse preneoplastic lesions in all mammary glands receive an injection of 500  $\mu$ g anti-CD25 Ig ( $\circ$ , 22 mice) on days 2 and 5. Other mice were electroporated on the first day of weeks 10 and 12 with the empty ( $\diamond$ , 22 mice) or EC-TM plasmid ( $\blacksquare$ , 20 mice), whereas other mice were electroporated with the EC-TM plasmid and injected with anti-CD25 Ig ( $\bullet$ , 29 mice). Mice of the same age were randomly assigned to the concurrently treated control and experimental groups. Each experiment was repeated two to four times, and the results were cumulated. *A*, Tumor incidence in mice receiving EC-TM plasmid electroporation combined with anti-CD25 Ig is significantly different ( $p < 0.0001$ ) as compared with all other groups. Tumor incidence was significantly delayed in mice injected with anti-CD25 Ig alone ( $p < 0.0001$ ), electroporated with EC-TM plasmid, and injected with anti-CD25 Ig ( $p < 0.0001$ ) as compared with untreated mice (data not shown) or mice electroporated with the empty pcDNA3 plasmid. Tumor incidence in groups of 10 mice untreated and electroporated with empty pcDNA3 plasmid combined with the two infusions of anti-CD25 Ig was not significantly different from that in mice injected with anti-CD25 Ig alone, and the data are not shown. *B*, Development of the titer of anti-rErbB2 Abs evaluated in individual mice. Horizontal lines, titer mean. *C*, Development of cytotoxic in vivo response against dominant p63–71 rErbB2 peptide. Horizontal lines, geometric mean. Only mice that were still tumor-free were tested.

with anti-CD25 Ig. To find evidence of this difference, we tested the usage of V $\beta$ 1–J $\beta$ 1.1, V $\beta$ 6–J $\beta$ 2.7, V $\beta$ 7–J $\beta$ 1.2, and V $\beta$ 9–J $\beta$ 1.2 in BALB/c and BALB-neuT mice electroporated with EC-TM plasmid at weeks 10 and 12 only, injected twice with anti-CD25 Ig at week 10 only, or electroporated and injected. Irrespective of the administration or not of anti-CD25 Ig, all the electroporated BALB/c mice expanded the V $\beta$ 9–J $\beta$ 1.2 rearrangements, although it was not used by any BALB-neuT mouse (Table I). Somewhat surprisingly, anti-CD25 Ig administration alone led to the expansion of V $\beta$ 1–J $\beta$ 1.1, V $\beta$ 6–J $\beta$ 2.7, and V $\beta$ 7–J $\beta$ 1.2 rearrangements in a limited but substantial number of BALB-neuT mice. These re-

arrangements are also expanded in most of BALB-neuT mice receiving the EC-TM plasmid electroporation plus anti-CD25 Ig administration.

#### Features of p63–71-specific T cells in BALB-neuT mice

The cytotoxic response elicited in vivo by the combined treatment in BALB-neuT mice is of lower avidity than what was induced in BALB/c mice electroporated with EC-TM only. Marked cytotoxic activity in vivo is evident against target cells only when these are pulsed with 15  $\mu$ M but not 3  $\mu$ M p63–71 (Fig. 4A). The cytotoxic response is parallel to a sustained CD8<sup>+</sup> T cell infiltration of mammary lesions in mice receiving the combined treatment (Fig. 4B). Accordingly, TCR repertoire analysis shows that CD8<sup>+</sup> cells expressing the V $\beta$ 1–J $\beta$ 1.1 and V $\beta$ 7–J $\beta$ 1.2 rearrangements infiltrate tumor lesions (Fig. 5B).

In vitro, too, the T cell response elicited by the combined treatment is of a relatively low avidity. Although a significant number of CD8<sup>+</sup> T cells from EC-TM–electroporated BALB/c mice is induced to produce IFN- $\gamma$  by 3  $\mu$ M p63–71, 15  $\mu$ M p63–71 is required to trigger CD8<sup>+</sup> IFN- $\gamma$ -secreting cells (Fig. 5A) from BALB-neuT mice receiving the combined treatment. Furthermore, the number of IFN- $\gamma$ -producing T cells in these mice is also consistently lower than that in BALB/c mice (Fig. 5), even under optimal concentration of stimulating Ag. The CD8<sup>+</sup> T cells carrying the shared rearrangements differ in their ability to expand in vitro in response to p63–71 (Fig. 5B). Those carrying the V $\beta$ 9–J $\beta$ 1.2 rearrangements used by EC-TM–electroporated BALB/c mice expand significantly already at a concentration of 3  $\mu$ M p63–71; of the rearrangements used by BALB-neuT mice receiving the combined treatment, V $\beta$ 7–J $\beta$ 1.2 alone displays a marginal ability to expand in the presence of 3  $\mu$ M p63–71. At the optimal concentration of 15  $\mu$ M p63–71, all of the three CD8-associated rearrangements expanded but with a lower proliferative capacity than the V $\beta$ 9–J $\beta$ 1.2 cells, as indicated by the lower RSI (Fig. 5B). Though not a direct measurement, the sensitivity to Ag stimulation in vitro has been shown to reflect the Ag avidity of a T cell (29). Thus, taken together, these results suggest that the CD8<sup>+</sup> cells activated in BALB/c mice constitute a repertoire characterized by high Ag avidity, whereas the CD8<sup>+</sup> cells recruited in BALB-neuT mice behaves as a repertoire characterized by lower avidity.

Last, the combined treatment is still able to elicit a reaction that impairs the progression of ErbB2 lesions in BALB-neuT/BKO mice unable to produce Abs (Fig. 4C). In the same way, depletion of CD8<sup>+</sup> cells reduces its ability to protect BALB-neuT mice from a subsequent tumor challenge (Supplemental Table II).

#### Anti-CD25 Ig administration expands but does not change CD4<sup>+</sup> T cell repertoires

We have previously shown that BALB-neuT mice expand CD4<sup>+</sup> T cells expressing the V $\beta$ 11–J $\beta$ 2.7 and the V $\beta$ 13–J $\beta$ 2.3 rearrangements following EC-TM electroporation, whereas BALB/c mice expand CD4<sup>+</sup> cells with different TCR rearrangements (15). To determine whether EC-TM plasmid electroporation plus anti-CD25 Ig also affects the CD4<sup>+</sup> T cell repertoire, LN cells from BALB/c and BALB-neuT mice electroporated with or without anti-CD25 Ig were cultured with rErbB2<sup>+</sup> BALB/c 3T3-NKB cells (23). Expansion of the V $\beta$ 11–J $\beta$ 2.7 and the V $\beta$ 13–J $\beta$ 2.3 rearrangements was observed following EC-TM plasmid electroporation with or without anti-CD25 Ig administration (Table I). Administration of anti-CD25 alone led to the expansion of lymphocytes expressing the V $\beta$ 11–J $\beta$ 2.7 and the V $\beta$ 13–J $\beta$ 2.3 rearrangements in a significant number of BALB-neuT mice. These results suggest that CD4<sup>+</sup> T cells expressing a public TCR repertoire specific for rErbB2 are naturally present in the periphery of BALB-

neuT mice and can be expanded either by active immunization or by T<sub>reg</sub> cell depletion alone. These rearrangements were not expanded in BALB/c mice receiving anti-CD25 Ig alone or in combination with EC-TM plasmid electroporation.

*Anti-CD25 Ig administration enables EC-TM plasmid electroporation to cure diffuse and invasive microscopic Erbb2 carcinomas*

To determine whether combination with anti-CD25 Ig improves the therapeutic efficacy of EC-TM plasmid, BALB-neuT mice received two courses of EC-TM plasmid electroporation, alone or combined with anti-CD25 Ig, at different stages of mammary cancer progression. Because T<sub>reg</sub> cells rapidly expand as a tumor grows, anti-CD25 Ig was injected 14 and 10 d before the first electroporation to remove most of them. At week 16, when multiple in situ carcinomas formed of clusters of tumor cells separated by bands of delicate stroma rich in blood vessels are scattered in all mammary glands (Supplemental Fig. 1), EC-TM plasmid electroporations alone still elicited a significant anti-rErbb2 Ab response but not a cytotoxic response, and the influence of this reaction on survival was still significant (Supplemental Fig. 1). Combination of anti-CD25 Ig led to a stronger Ab response, triggered a significant cytotoxic response to the dominant p63–71 (Fig. 6), reduced tumor multiplicity (Supplemental Table I), and kept 36% of mice free of palpable tumors at week 100 when the experiment ended (Fig. 6).

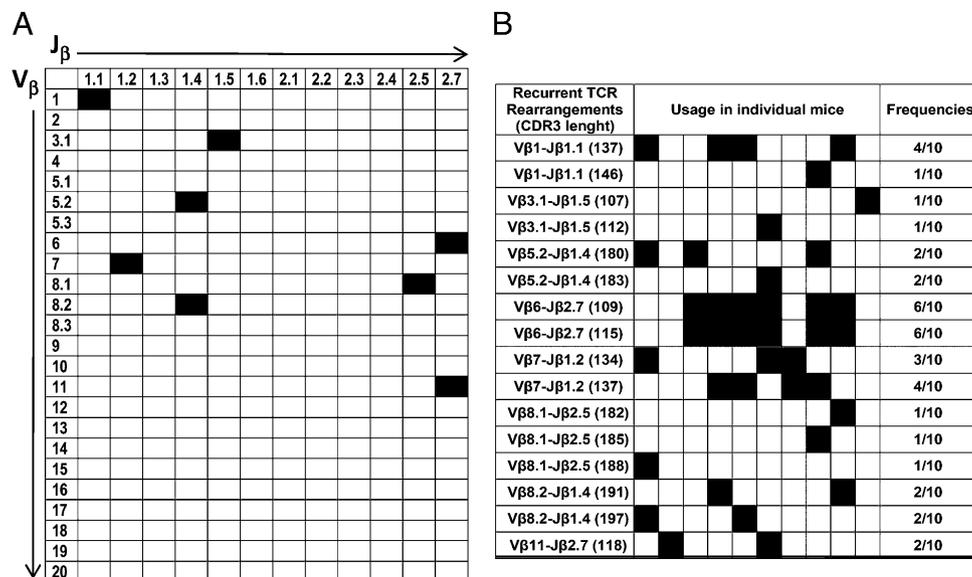
At week 18, the clusters coalesce to form masses that invade mammary glands (Fig. 6) and are surrounded by a dense stroma with large vessels (Supplemental Fig. 1). Once again, EC-TM plasmid electroporation alone elicited a significant Ab response, but its influence on survival was marginal (Supplemental Fig. 1). By contrast, the combination continued to elicit a stronger Ab and cytotoxic response and kept 33% of mice free of palpable tumors (Fig. 6) and alive (Supplemental Fig. 1) at the end of the experiment.

At week 20, larger microscopic masses start to become palpable in the mammary pad (Fig. 6). They often display central necrotic areas (Supplemental Fig. 1) because blood vessels are only present in the surrounding dense and fibrous stroma. These vessels show a diffuse NG2 and  $\alpha$ -smooth muscle actin (markers of nascent and mature pericytes) positivity, which is absent and scarcely present in the tumor vessels of 16- and 18-wk-old mice, respectively (Supplemental Fig. 2). At this stage, EC-TM plasmid electroporation elicited only a low Ab response, whereas its combination with anti-CD25 Ig led to a slightly higher Ab response together with a substantial cytotoxic response that significantly delayed the appearance of the first palpable tumor (Fig. 6) and appreciably reduced both tumor multiplicity (Supplemental Table I) and total tumor burden (Supplemental Fig. 3), although the extension of survival was marginal (Supplemental Fig. 1).

## Discussion

BALB-neuT mice are genetically predestined to undergo one of the most aggressive metastasizing and lethal rErbb2-driven mammary carcinogenesis (11, 12). During the lengthy stepwise progression of their mammary lesions, T<sub>reg</sub> cells expand to bring about rampant immunosuppression (19). When the lesions are initial and the tumor-elicited suppression is still negligible, the protection provided by anti-rErbb2 vaccines prevents their progression and persists for almost as long as the natural life span of wild-type BALB/c mice (14, 22), whereas their efficacy dramatically fades if these initial lesions have progressed, and is almost null against diffuse invasive microscopic tumors (14, 22, 23, 30).

We now report new findings that give insight into the unveiling of latent CD8<sup>+</sup> T cell populations, the therapeutic efficacy stemming from the combination of anti-Erbb2 DNA vaccination with anti-CD25 Ig administration, and the limit of this combined treatment in advanced microscopic tumor stages.



**FIGURE 3.** Dominant rErbb2 peptide T cell repertoires triggered by EC-TM plasmid electroporation and anti-CD25 Ig administration in BALB-neuT mice. **A**, Complete CDR3- $\beta$  immunoscope analysis of the immune response to p63–71 peptide in a pool of five BALB-neuT mice. Mice were electroporated on weeks 10 and 12 with EC-TM plasmid and injected with anti-CD25 Ig 1 and 4 d after the first electroporation. Their popliteal and inguinal LN cells were restimulated in vitro with the immunodominant rErbb2 peptide p63–71. cDNA were amplified by PCR with a combination of all 24 V primers and a common C primer; after a runoff reaction with 12 J fluorescent primers, products were analyzed in a DNA sequencer. ■, V-J rearrangements showing p63–71-specific expansion. **B**, Frequencies of the specific p63–71 peptide repertoires in 10 individual mice electroporated and treated with anti-CD25 Ig as in **A**. Each column shows one mouse; ■, rearrangements used by individual mice. Frequencies of usage of the rearrangements are indicated as number of mice that use that recombination per total mice.

Table I. Usage of T cell repertoire rearrangements in BALB/c and BALB-neuT mice electroporated with EC-TM plasmid and/or treated with anti-CD25 Ig

Mice	Treatment	Vβ6-Jβ2.7 (%) <sup>a</sup>	Vβ1-Jβ1.1 (%) <sup>a</sup>	Vβ7-Jβ1.2 (%) <sup>a</sup>	Vβ9-Jβ1.2 (%) <sup>a</sup>	Vβ11-Jβ2.7 (%) <sup>b</sup>	Vβ13-Jβ2.3 (%) <sup>b</sup>
BALB/c	EC-TM	0/5 <sup>c</sup> (0)	0/5 (0)	0/5 (0)	5/5 (100)	0/5 <sup>b</sup> (0)	0/5 (0)
	Anti-CD25Ig	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	EC-TM + anti-CD25Ig	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)	0/5 (0)	0/5 (0)
BALB-neuT	EC-TM	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)	5/5 (100)
	Anti-CD25 Ig	3/8 (38)	3/8 (38)	4/8 (50)	0/8 (0)	2/4 (50)	1/4 (25)
	EC-TM + anti-CD25Ig	9/14 (64)	7/14 (50)	9/14 (64)	0/14 (0)	14/14 (100)	14/14 (100)

<sup>a</sup>TCR rearrangements expanded following p63–71 ErbB2 peptide in vitro restimulation.

<sup>b</sup>TCR rearrangements expanded in LN cells from variously treated mice recovered after in vitro restimulation with rErbB2<sup>+</sup> 3T3-NKB cells.

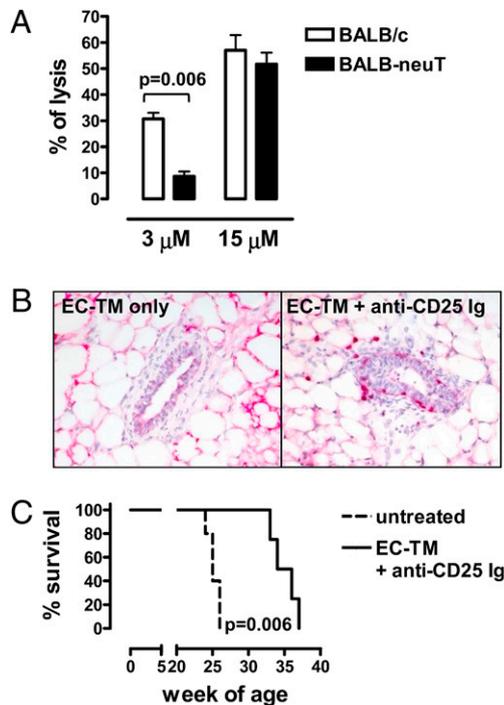
<sup>c</sup>Mice using the indicated TCR rearrangement/number of total analyzed mice.

First, in BALB-neuT mice on the threshold of mammary carcinogenesis (10 wk), two administrations of anti-CD25 Ig lead to a rapid depletion of CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>+</sup>FoxP3<sup>+</sup>T<sub>reg</sub> cells followed by a slow return to the levels of untreated mice. This depletion alone delays the appearance of the first palpable tumor, although all mice have one or more tumors by week 27.

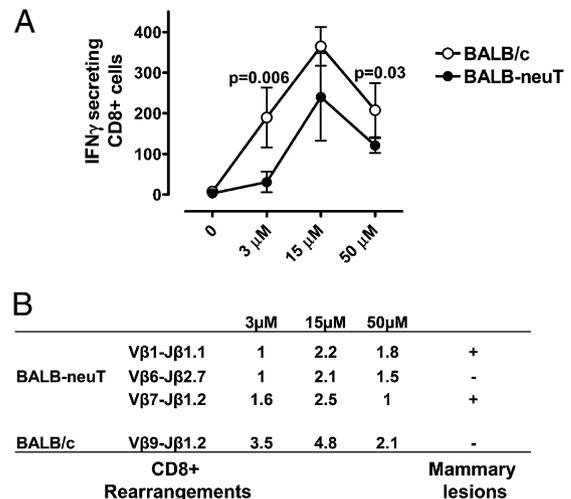
Second, the finding that combination of vaccination with anti-CD25 Ig triggers a significant cytotoxic response to the rErbB2 dominant peptide in BALB-neuT mice was unexpected. Our previous work showed that a significant cytotoxic response was

never achieved in BALB-neuT mice by successful immunizations with vaccines of different kinds (13, 14, 16, 22, 28, 31). Newborn BALB-neuT mice, in fact, express rErbB2 in the thymus, and central tolerance appears to delete the CD8<sup>+</sup> T clones that interact at high avidity with the dominant H-2K<sup>d</sup>rErbB2 peptide in BALB/c mice. Analysis of the TCR repertoire of T cells expanded following anti-rErbB2 DNA immunization showed that BALB-neuT mice lack these clones, whereas they are expanded in wild-type BALB/c mice (15).

Vβ-Jβ spectratyping shows that a low-avidity cytotoxic response is induced by vaccination plus anti-CD25 Ig; this rests on expansion of CD8<sup>+</sup> T cells belonging to an otherwise cryptic TCR repertoire that do not expand in wild-type BALB/c. In these mice, anti-rErbB2 immunization, alone or in combination with anti-CD25 Ig, expands only high-avidity CD8<sup>+</sup> cytotoxic T cells. These data fit in well with our previous demonstration that wild-type BALB/c and transgenic BALB-neuT mice use distinct and nonoverlapping TCR repertoires in response to rErbB2 vaccination (15). T<sub>reg</sub> cell depletion does not revert the clonal composition of the CD8<sup>+</sup> TCR repertoire specific for the dominant rErbB2 peptide

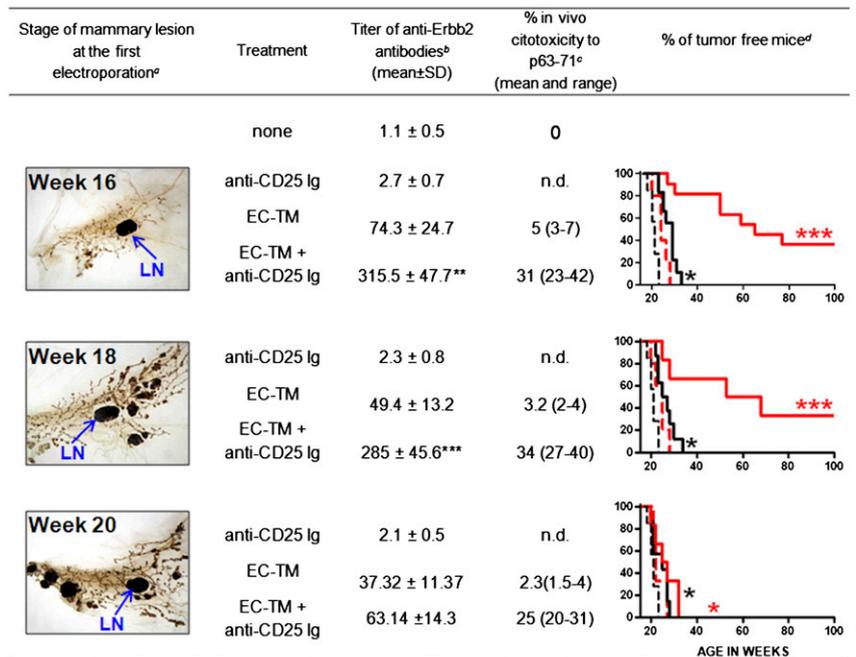


**FIGURE 4.** p63–71-specific T cells are elicited by the combined treatment. Features of the T cell reactivity elicited in BALB/c, BALB-neuT, and BALB-neuT/BKO mice electroporated on weeks 10 and 12 with EC-TM plasmid and injected or not with anti-CD25 and tested on week 14. *A*, In vivo cytotoxicity against cells pulsed with 3 or 15 μM p63–71. □, BALB/c mice electroporated with EC-TM only; ■, BALB-neuT mice electroporated with EC-TM and injected with anti-CD25. A representative experiment of three performed independently is shown. *B*, Staining of CD8<sup>+</sup> cells (in purple) close to mammary ducts in BALB-neuT mice electroporated with EC-TM only (*left panel*) and those receiving the combined treatment (*right panel*) (original magnification ×40). CD8<sup>+</sup> cells infiltrating mammary glands counted ~40 ducts per group were as follows: 1.65 ± 0.9 (mean ± SD) in mice receiving the combination treatment and 0.68 ± 0.28 in those electroporated with EC-TM only (*p* < 0.0001). *C*, Tumor incidence in BALB-neuT/BKO mice untreated (five mice) and receiving the combined treatment (four mice).



**FIGURE 5.** p63–71-specific T cells are of low avidity. *A*, IFN-γ production by CD8<sup>+</sup> LN cells restimulated with 3, 15, and 50 μM p63–71. Mean ± SD of the number of positive cells/10<sup>5</sup> cells. BALB/c mice electroporated with EC-TM on weeks 10 and 12 (○); BALB-neuT mice electroporated with EC-TM at weeks 10 and 12 and injected with anti-CD25 Ig (●). CD8<sup>+</sup> LN cells were tested on week 14. Horizontal bars: means. Statistic compares BALB-neuT versus BALB/c. *B*, RSI of shared TCR rearrangements used by CD8<sup>+</sup> LN cells of BALB-neuT (Vβ1-Jβ1.1, Vβ6-Jβ2.7, and Vβ7-Jβ1.2) and BALB/c mice (Vβ9-Jβ1.2) restimulated at the same concentration of p63–71 as above and by T cells infiltrating mammary lesions. Results are indicated as average RSI, where RSI of 1 indicates no stimulation.

**FIGURE 6.** Anti-CD25 enhances the therapeutic activity of EC-TM electroperoration in BALB-neuT mice. At progressive stages of mammary carcinogenesis, BALB-neuT mice received EC-TM electroperoration alone or combined with anti-CD25 Ig injected 14 and 10 d before the electroperoration course. The pictures in the *left panels* show mammary gland whole-mount preparations performed in unvaccinated mice at 16, 18, and 20 wk of age. At 16 wk, the mammary gland displays multiple small opacities surrounding the mammary ducts indicating the presence of diffuse atypical hyperplastic lesions and several foci of in situ carcinoma. At 18 wk, several discrete dark nodules corresponding to in situ and early invasive carcinomas are present, whereas the larger nodules with irregular borders present at 20 wk are interpretable as solid invasive carcinomas. The central oval black areas are mammary LNs. Original magnification  $\times 6.3$ .



<sup>a</sup>whole mount images ( $\times 6.3$ ).

<sup>b</sup>Sera collected two weeks after the second electroperoration. Titers expressed as s.b.p. $\times 10^3$ .

<sup>c</sup>Cytotoxic activity evaluated in groups of additional three mice sacrificed two weeks after the second electroperoration. Mean values of a representative experiment of three performed independently are shown.

<sup>d</sup>Incidence of tumor in BALB-neuT mice: dotted black lines: mice electroperated with empty plasmid; dotted red lines: mice injected with anti-CD25; black lines: mice electroperated with EC-TM only; red lines: mice receiving both treatment. Statistical analysis of survival: red asterisks, mice receiving EC-TM and anti-CD25 versus those receiving EC-TM only; black asterisks, mice electroperated with EC-TM only versus those receiving the empty plasmid. \*\*\*,  $p < 0.0001$ ; \*\*,  $p < 0.001$ ; \*,  $p < 0.05$ .

toward that of wild-type BALB/c mice. Instead, it unveils a latent repertoire of rErbB2-specific CD8<sup>+</sup> T cells seemingly exclusive of the BALB-neuT mice. The low avidity of these cells may underlie their cryptic behavior. Although low-avidity cells can escape thymus selection (10, 32), their access to Ag is possibly limited by competition with T cells of higher avidity in BALB/c mice (33–35). Alternatively, it can be suggested that the cryptic CD8<sup>+</sup> repertoire is positively selected only in BALB-neuT mice as an effect of thymic expression of rErbB2 (15). In these mice, the cryptic repertoire appears to be concealed by T<sub>reg</sub> cells expanded following rErbB2 overexpression in the thymus and/or in neoplastic lesions. Reversal of its tolerogenic state by T<sub>reg</sub> cell depletion allows it to expand and display a cytotoxic response. T<sub>reg</sub> cells also hinder the spontaneous expansion of CD4<sup>+</sup> T cells specific for rErbB2. Expansion of the V $\beta$ 11-J $\beta$ 2.7 and V $\beta$ 13-J $\beta$ 2.3 CD4<sup>+</sup> T cell repertoires following the administration of anti-CD25 Ig alone suggests that they are naturally present in the periphery of BALB-neuT mice but are suppressed by the expanded T<sub>reg</sub> cells.

Third, our data also illustrate the importance of counteracting T<sub>reg</sub> cell expansion in obtaining a strong Ab response and the induction of a cytotoxic response during cancer progression and thus endorse findings in studies of tumors transplanted in syngeneic mice (36) and in ErbB2<sup>+</sup> tumors transplanted in rErbB2 transgenic mice (5). The inhibitory activity of tumor-expanded T<sub>reg</sub> cells acts on both CD8<sup>+</sup> and CD4<sup>+</sup> lymphocytes. Adoptive transfer experiments (22, 23) and experiments with BALB-neuT mice rendered deficient in immune components through gene targeting or Ab-mediated depletion of selected lymphocyte populations (16, 22, 23, 28, 31, 37) have made it clear that Abs elicited by anti-rErbB2 vaccination are both necessary and sufficient for protection insofar because they impede neoplastic proliferation by directly downregulating rErbB2 receptor expression, confining it in the cytoplasm and impeding the formation of

homo- or heterodimers that transduce proliferative signals (16, 28), and triggering cellular cytotoxicity (38). By competing in the binding to APCs, T<sub>reg</sub> cells inhibit the expression of costimulatory molecules (39). Their depletion thus results both in the improved costimulatory activity of APCs and in a stronger Ab- and cell-mediated response. The better efficacy stemming from the combination of vaccination and anti-CD25 Ig thus rests on both higher Ab response and activation of the new CD8<sup>+</sup> T cell population reacting with low avidity with rErbB2 that secretes IFN- $\gamma$  and effectively kills p63–71-pulsed cells. In several mouse models, low-avidity T cells alone are enough to suppress the growth of the tumor expressing the Ag (40, 41). Collaboration of the humoral and the cellular immune responses in rejection of rErbB2 tumors in transgenic mice has been documented (42). In addition, our previous studies in BALB-neuT mice receiving the adoptive transfer of Abs and T cells have shown the importance of both humoral and cellular reactivity in hampering the progression of mammary cancer (22, 24). In BALB-neuT mice receiving an ErbB2 vaccine alone, the role of CD8<sup>+</sup> T cells in protecting against rErbB2 tumors is negligible (13, 17, 28). By contrast, the importance of the cytotoxic activity appearing after T<sub>reg</sub> cell removal is shown by the fact that depletion of CD8<sup>+</sup> T cells reduces the ability of the combination of vaccine and anti-CD25 Ig to protect BALB-neuT mice from a lethal challenge of ErbB2<sup>+</sup> tumor cells. Moreover, in BALB-neuT mice knocked out for the  $\mu$ -chain of the Ig and thus unable to produce Abs following rErbB2 vaccination, the cellular reactivity triggered by the combined vaccination is enough to delay the progression of ErbB2 mammary lesions, even if it does not provide a long lasting protection against the development of autochthonous tumors. This, in fact, requires the presence of anti-ErbB2 Abs.

The stepwise progression of mammary cancer in BALB-neuT mice has been exploited to show the greater efficacy of vaccination plus T<sub>reg</sub> cell depletion at tumor stages at which vaccination alone

is ineffective. In mice bearing an atypical hyperplasia in all mammary glands, our anti-rErbB2 DNA vaccine alone doubles the survival time, but by the 65th week of age, all mice have developed tumors. Moreover, although the efficacy of the DNA vaccine alone is no more than marginal in mice bearing diffuse in situ carcinoma (16th week of age) and early microscopic invasive cancer (week 18), its combination with anti-CD25 Ig was still able to keep a significant number of mice tumor free at week 100, whereas in those that developed tumors, tumor multiplicity was significantly reduced. This is probably the most significant finding of this study.

Fourth, even the more composite and effective immune response induced by the combination of anti-rErbB2 vaccination with anti-CD25 Ig loses most of its efficacy as the microscopic lesions progress. Survival, in fact, is no more than marginally extended if this combination is administered when they are nearly palpable in the mammary pad (20th wk of age), because it does no more than slow down the progress of a few lesions and reduce the number of tumors per mouse. This loss of efficacy is probably due to increased resistance acquired by the more advanced neoplastic lesions as they become more compact and refractory to the immune mechanisms thus activated (43, 44). The growth of these lesions is fatal, even if the expansion of smaller lesions is still controlled by the immune response. However, our data also point to a marked decrease of the intensity of the immune response elicited by the combination in mice bearing invasive microscopic cancer. Although anti-CD25 Ig still results in T<sub>reg</sub> cell deletion (data not shown), this interference with negative regulatory cells is no longer enough to induce a marked Ab response. As a tumor expands, additional mechanisms such as PGE<sub>2</sub>-induced, tumor-associated macrophages and IDO-producing dendritic cells (6, 7, 9) may acquire a dominant role in suppressing the immune response.

In conclusion, this study shows that temporary deletion of T<sub>reg</sub> cells in combination with anti-rErbB2 vaccination enhances Ab production and activates a latent population of cytotoxic CD8<sup>+</sup> T cells expressing a cryptic repertoire. The immune response thus generated is of therapeutic significance in that it halts the progression of lesions that cannot be inhibited by vaccination alone. These results underscore the importance of using optimal vaccine strategy when targeting tumor Ags (5, 45). However, our data also indicate that caution is needed when assessing the significance of vaccination plus T<sub>reg</sub> cell deletion in a clinical setting, because it is quite possible that the long-lasting tumor host relationship that characterize human tumors will minimize the immune advantage because of such depletion unaccompanied by attempts to counter other forms of immune suppression. Moreover, even though BALB-neuT mice reproduce several features of human breast cancer, one cannot rule out the possibility that the different patterns and timing of transgene expression lead to an immune tolerance to ErbB2 different from that of tumor patients (1).

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## Disclosures

The authors have no financial conflicts of interest.

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