Peptide Vaccination Breaks Tolerance to HER-2/neu by Generating Vaccine-Specific FasL+ CD4+ T Cells: First Evidence for Intratumor Apoptotic Regulatory T Cells

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Abstract

BALB/c mice transgenic (Tg) for the transforming rat neu oncogene (BALB-neuT) are genetically predestined to develop mammary carcinogenesis in a process similar to that in humans. We crossed HLA-A2.1/HLA-DR1 (A2.1/DR1) Tg mice with BALB-neuT mice to generate A2.1/DR1 × BALB-neuT triple Tg (A2.1/DR1 × neuT+) mice, which represent an improvement over BALB-neuT mice for evaluating vaccination regimens to overcome tolerance against HER-2/neu. A vaccine formulation strategy, consisting of synthetic peptides from the rat HER-2/neu oncogene combined with granulocyte macrophage colony-stimulating factor, was highly effective in preventing the growth of established transplantable tumors in male A2.1/DR1 × neuT+ mice. Vaccination with HER-2(435–443) (p435) CTL peptide alone induced weak antitumor responses, which were characterized by increased numbers of regulatory T cells (Treg) and low numbers of vaccine-specific CD8+ CTL and helper T cells (Th). The administration of p435 plus HER-2(776–790) (p776; helper peptide) reversed this situation, inducing functionally active, peptide-specific CTL and Th. There was a striking change in the intratumoral balance of Tregs (decrease) and vaccine-specific Th (increase) that directly correlated with tumor rejection. Intratumoral administration of anti-FasL antibody promoted tumor growth. The decrease in Tregs (Fas+) was due to apoptosis induced by cell contact with Fas ligand (+) Th. Mice vaccinated with p435 plus p776 exhibited long-lasting antitumor immunity. Our vaccine regimen also significantly delayed the outgrowth of mammary carcinomas in female A2.1/DR1 × neuT+ animals. We provide a mechanism to overcome tolerance against HER-2/neu, which proposes a combined vaccination with two (Th and CTL) HER-2 peptides against HER-2/neu–expressing tumors. Cancer Res; 70(7); 2686–96. ©2010 AACR.

Introduction

HER-2/neu oncoprotein is overexpressed by a significant percentage of human carcinomas (1), and this, at least in breast cancer, is associated with aggressive disease and enhanced metastatic potential (2). Overexpression of HER-2/neu poses a significant challenge to the design of effective cancer vaccines because it is recognized as a “self” molecule, and this implies that tolerance mechanisms against HER-2/neu must be circumvented. Based on HER-2/neu transgenic (Tg) mouse models (3, 4), we now know that T cells specifically recognizing an immunodominant epitope from the transgene (self) are rare and of lower avidity (4, 5). These low-avidity T cells, upon efficient costimulation, have the capacity to develop antitumor responses (6–8). However, higher-avidity T cells specific for epitopes encoded by the transgene can escape thymic deletion and are actively suppressed in the periphery of these mice by CD4+CD25+ regulatory T cells (Treg; refs. 5, 8). We (9) and others (4, 7, 8, 10, 11) have shown that removal of Tregs by various means has led to generation of antitumor immunity. Thus, the observation that T cells recognizing (self) tumor antigens persist in vivo suggests that elimination through tolerance is not complete and underscores the importance of defining potent vaccines specifically activating these cells.

Here, we evaluate the use of a vaccine mixture consisting of two immunogenic peptides corresponding to the HER-2(776–790) (p776) CTL epitope (12–16) and the promiscuous p776 helper T cells (Th) epitope (17–21).

This is the first study, to our knowledge, defining the in vivo effect of combined vaccination with two HER-2/neu peptides representing CTL and Th epitopes on the dynamic relationship between vaccine-specific CTL and Th/Tregs in HER-2/neu tolerant A2.1/DR1 × neuT+ and nontolerant A2.1/DR1 × neuT− littermates. In addition, we show that co-vaccination with p776 induces peptide-specific FasL+ Th, which, via cell contact, induce apoptosis in Fas− Tregs within the tumor microenvironment. Our data strongly suggest that the therapeutic effect of this vaccine combination resides in...
increases in the intratumor frequency and activity of peptide-specific CTL and Th as a consequence of priming of this type of lymphocytes and removal of Tregs. Finally, the data also show that our vaccination schedule induces memory antigen immunity, which is important considering the use of this peptide combination in clinical studies.

Materials and Methods

Generation of triple Tg mice. Female A2.1/DR1 mice (ref. 22; provided by Prof. F.A. Lemonnier; Unite d’Immunite Cellulaire Antivirale, Institute Pasteur) were crossed with male neuT mice (ref. 3; provided by Prof. G. Forni; Molecular Biotechnology Center, University of Turin), and ∼30% of the progeny expressed the neu transgene (A2.1/DR1 × neuT triple Tg), as verified by multiplex PCR. Experiments for this study were approved by the Saint Savas Institutional Animal Care and Use Committee.

Synthetic peptides. HER-2(435–443) (p435; ILHDGAYSIL) and p776 (GVSPYYSRLLGICL) peptides were synthesized and analyzed as described in our previous reports (13, 19).

Antibodies. Antibodies used were as follows: anti-CD107α-FITC, anti-IFNγ-FITC, anti-CD95-FITC (anti-FAS), anti-CD178-PE (anti-FAS ligand), anti-CD127-PE, anti-CD8-APC, anti-Foxp3-APC, and anti-Foxp3-PerCP-Cy5.5 (eBioscience); anti-HLA-A2.1 (a gift from Prof. H.G. Rammensee, Department of Immunology, University of Tuebingen); PE-labeled HLA-A2.1 pentamer presenting the p435 epitope (PENT; Proimmune, Magdalen Centre, Oxford Science); and PE-labeled HLA-DR1 tetramer presenting the p776 epitope (Tm; Beckman Coulter).

Immunoization protocol. Mice were immunized thrice every 5 d by s.c. injections at the base of the tail with 100 μg of peptide in 200 μL incomplete Freund’s adjuvant (IFA) along with 100 μg of granulocyte macrophage colony-stimulating factor (GM-CSF) as described (9, 13).

In vitro cytotoxicity assay. CD8+ T cells were isolated from immunized animals (9) and used as effectors against the indicated targets, as recently described (9, 13). The HER-2 TUBO mammary carcinoma cell line (5) was transfected to express HLA-A2.1 (TUBO.A2) by a method described in ref. (13).

Therapeutic tumor models. (A) Mice were inoculated s.c. with 5 × 10⁶ transplantable TUBO.A2 cells. Seven days later, peptides (single or in combination) in IFA were injected along with GM-CSF, as described (9, 13). To test the capacity of the peptide combination vaccine to induce antitumor memory responses, mice were first vaccinated as above and, 8 wk later, were challenged with TUBO.A2 cells. Tumor size was monitored regularly every 4 d and was expressed as the product (in mm³) of the perpendicular diameters of individual tumors. In some experiments, mice were treated intratumorally (days 30–40 after tumor inoculation) once with 200 μg of a blocking anti-Fasl monoclonal antibody (mAb; IgG1 antitrustomous CD178, clone MFI3; BD Biosciences) or an isotype-matched control mAb (clone A19-3; BD Biosciences) in 100 μL PBS.

(B) Six-week-old female A2.1/DR1 × neuT triple Tg mice were vaccinated as above. Vaccinations were repeated monthly for 3 subsequent months (i.e., until these animals were 18 wk old). Mammary glands were inspected at weekly intervals to note tumor appearance. Progressively growing masses (mean diameter, >2 mm) were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of incident tumors per total number of mice and was reported as mean ± SD (8).

Isolation of tumor-infiltrating lymphocytes. This was essentially performed as recently described (23).

Surface and intracellular staining. For surface staining, cells were incubated with mAb in different combinations on ice for 30 min. For Foxp3, IFNγ (intracellular), and Annexin V′ staining, the manufacturers’ instructions were followed. CD8′PENT′ anti-CD107α-FITC′anti-IFNγ-FITC′anti-CD95-FITC′ and CD4′Th′ were detected as recently described (9). Stained cells were analyzed using a FACS Calibur (BD Biosciences).

Treg and Th tumor-infiltrating lymphocyte isolation and cultures. CD4′CD25highCD127−CD4′CD25high′CD127+ tumor-infiltrating lymphocytes (TIL) in combination with an anti-PE multisort kit (Miltenyi Biotec; ref. 24). Thereafter, CD127-depleted cells were used for positively selecting CD4′CD25high′′CD127−′ cells using the CD4′CD25′ Treg isolation kit (Miltenyi Biotec) according to manufacturer’s protocol. The MACS-sorted CD4′CD25high′′CD127−′′′ Tregs were >95% pure, as determined by fluorescence-activated cell sorting (FACS; Supplementary Fig. S1). CD4′CD25′ Th cells were isolated from the same tumors with MACS beads (Miltenyi Biotec; ref. 24). The purity of CD4′CD25′ Th was >99% (Supplementary Fig. S1). Equal numbers of Tregs and Th (2 × 10⁶ cells in 2 mL) were distributed in 200 μL aliquots per well in a 96-well round-bottomed plate (Costar). After 24 h in a CO₂ incubator, cells were collected, stained with mAb, and analyzed by FACS.

Statistical analyses. The Student’s t test was applied with a 95% confidence interval to determine the statistical significance of differences between groups, with P < 0.05 being considered significant.

Results

Immunogenicity of the peptide combination vaccine in HER-2/neu tolerant A2.1/DR1 × neuT triple Tg. Our first task was to determine the immunogenicity of p435 in our HER-2/ neu tolerant male A2.1/DR1 × neuT triple Tg (hereinafter called neuT′ triple Tg) versus their HER-2/neu nontolerant littermates (A2.1/DR1 × neuT′; hereinafter called neuT′ littermates) given either alone or in combination with p776. Two major findings emerged from these studies (Fig. 1Aa): firstly, only marginal levels of cytotoxicity could be detected in neuT′ triple Tg when p435 was given alone. In contrast, this peptide elicited strong cytotoxic responses in neuT′ littermates (P < 0.001). Secondly, the levels of cytotoxicity in neuT′ triple Tg were greatly enhanced when p435 was given...
Figure 1. In vitro CTL activity (Aa) and flow cytometric HLA-A2/p435 pentamer (PENT+) analyses (Ab) of CD8+ T lymphocytes isolated from spleens of male A2.1/DR1 × neuT+ triple Tg and A2.1/DR1 × neuT− littermates immunized with the indicated HER-2/neu peptides. Mean values ± SD from pooled data (four mice per group; Aa) and individual values (Ab). Male neuT− (B) or neuT+ (C) animals were challenged with TUBO.A2 transplantable tumors, followed by injection 7 d later with the indicated HER-2/neu peptides or PBS. Da, neuT+ tolerant triple Tg were first vaccinated and then challenged (8 wk later) with TUBO.A2 cells. Db, neuT+ mice were vaccinated only with p776. Dc, neuT+ mice were vaccinated as in Cc, and when tumor size was ~70 mm^2 (days 30–40 after tumor inoculation), mice were additionally treated intratumorally with 200 μg of an anti-FasL mAb (or with the isotype matched control mAb clone A19-3).
together with p776 (P < 0.001 versus vaccination with p435 alone). As expected, the combined administration of these peptides further improved CTL responses in neuT− littermates (P < 0.05 versus vaccination with p435 alone). Cytotoxicity levels were correlated with the number of p776-specific CD8+ T cells, defined as HLA-A2.1/HER-2(9435) pentamer+ (PENT+CD8+) cells, in the spleen (Fig. 1Ab). None of the mice (neuT+ or neuT−) injected with GM-CSF and IFA alone developed any detectable levels of cytotoxicity (data not shown).

**Combined vaccination with p435 and p776 induces potent antitumor responses in tumor-bearing neuT+ triple Tg.** We then sought to investigate whether this vaccine formulation could act therapeutically against growing TUBO.A2 cells in male neuT+ triple Tg. Animals were first inoculated with transplantable TUBO.A2 cells followed by injection of 7 days later with the indicated HER-2/neu peptides. In control (PBS) neuT+ mice, tumor grew fast and reached a size of ∼200 mm2 after 20 to 25 days (Fig. 1Ba). A moderate, but significant, delay of TUBO.A2 outgrowth was noticed in all seven neuT− littermates immunized with p435 alone (P < 0.05; Fig. 1Bb), whereas a much stronger effect, upon vaccination with the peptide mixture, was seen in these animals (P < 0.001; Fig. 1Bc). Tumor growth showed similar kinetics in control neuT+ triple Tg (Fig. 1Ca). However, vaccination with p435 had no effect on tumor growth in this group of animals (P < 0.05; Fig. 1Cb). In contrast, the majority of neuT+ mice (7 of 11) rejected their tumors upon immunization with the combination of p435 plus p776 (P < 0.001; Fig. 1Cc), whereas the remainders in this group showed a significant delay of tumor growth (P < 0.005; Fig. 1Cc). In addition, the combination vaccine had no effect on the growth of wild-type TUBO cells, which do not express HLA-A2.1 (data not shown). To evaluate the potency of our peptide combination vaccine to induce a protective antitumor memory response, neuT+ triple Tg were immunized with p435 plus p776 and challenged with TUBO.A2 cells 8 weeks later. As shown in Fig. 1Da, neuT+ triple Tg were protected as efficiently as in experiments wherein the combination vaccine was given 7 days after tumor inoculation (Fig. 1Cc), thus indicating that the antitumor T cells generated by the vaccine can last for at least 2 months. p776 was found to be immunogenic in naive neuT+ triple Tg generating IFNγ producing CD4+ T cells (Supplementary Fig. S2), yet it did not show any antitumor activity when used as a single-peptide vaccine in those animals inoculated with TUBO.A2 tumor cells (Fig. 1Db).

**p776 increases the percentages of functionally active p435-specific intratumor CD8+ T cells.** We next studied whether this type of treatment could have an effect on the frequencies of p435-specific CD8+ T cells infiltrating the growing tumors. Tumors from mice with progressively growing tumors were harvested at a time point when their size reached ∼70 mm2 (Fig. 1Bb and Cb); in mice which finally rejected their tumors, these were removed at an early time point, following the beginning of their regression (usually on days 30–40; see Fig. 1Bc and Cc). As shown in Fig. 2, upon vaccination with p435 alone, we could detect a significant number of PENT+CD8+ cells within the tumor microenvironment in both neuT+ triple Tg (Fig. 2Aa) and neuT− littermates (Fig. 2Bb). Notably, in neuT+ animals, the percentage of PENT+CD8+ TIL was significantly lower compared with what we observed in neuT− littermates (P < 0.01). We could also show that the vast majority of PENT+CD8+ TIL in neuT− littermates expressed high levels of intracellular IFNγ and membrane CD107a (Fig. 2Ba). Importantly, CD107a mobilization and IFNγ production were detected only in a minority of PENT+CD8+ TIL in neuT+ triple Tg (Fig. 2Aa).

Treatment with the combination vaccine (i.e., p435 plus p776) significantly increased the percentages of PENT+CD8+ TIL in both neuT− littermates and neuT+ triple Tg (Fig. 2Ab, Bb, and Cb; P < 0.005). Strikingly, in neuT+ triple Tg, almost all of the p435-specific CD8+ TIL were functionally active, as determined by intracellular IFNγ and membrane CD107a staining (Fig. 2Ab). Therefore, it seems that besides amplifying the anti-p435–specific CTL response the p776 vaccine also provides signals that reverse tolerance against HER-2/neu–specific CD8+ T cells in neuT+ triple Tg.

**Mice vaccinated with p435 plus p776 have increased CD4+ Foxp3+/CD4+ Foxp3− ratios in the tumor microenvironment.** It was important to find out whether p776 could induce changes in CD4+Foxp3− to CD4+Foxp3+ ratios within the tumor microenvironment and thereby potentiate antitumor immunity. When immunized with the CTL vaccine alone, neuT− triple Tg exhibited increased percentages of CD4+ Foxp3+ Tregs compared with their neuT+ littermates (P < 0.001; Fig. 3Aa and Ba). The vast majority of CD4+Foxp3+ Tregs in neuT− animals were CD25+ (Fig. 3Aa). The ratio of CD4+ Foxp3+ TIL to CD4+ Foxp3− Treg TIL was significantly lower in neuT+ triple Tg compared with neuT− littermates (Th/Treg ratios, 0.4 and 1.7, respectively; Fig. 3Aa and Ba). Interestingly, greater differences in the percentages of these CD4+ T-cell populations were observed after combined vaccination with p435 and p776. The Th/Treg ratio was increased up to 7-fold at the tumor site in neuT+ triple Tg (Fig. 3Ab and Cb). An increase of Th TIL was also revealed in neuT− littermates receiving the combination vaccine (Fig. 3Bb and Cb). Interestingly, the increase in Th/Treg ratios was associated with a decrease in Treg TIL (Fig. 3Ab and Bb). Analyses of splenocytes revealed increasing numbers of Tregs in neuT+ mice 15 days after vaccination with p435 (day 15 versus day 1 after vaccination, P < 0.005), which were significantly decreased (P < 0.001) upon vaccination with p435 plus p776 (Fig. 3Da). An analogous situation was also observed in neuT− littermates with a weak, but significant, increase in Tregs upon vaccination with p435 (day 15 versus day 1, P < 0.005) and a strong decrease of this population in the combined vaccination protocol (P < 0.001; Fig. 3Db). It is of importance to note that the percentages of Tregs in neuT+ triple Tg vaccinated with both peptides were significantly lower compared with Tregs in neuT− littermates vaccinated with the CTL peptide only (Fig. 3Ca versus Cb for TIL and Fig. 3Da versus Db for Tregs in the spleen measured 15 days after vaccinations; P < 0.005 for both cases).

**Intratumor Th specifically recognize p776.** To evaluate TCR specificity and functional status of Th infiltrating tumors in our vaccinated triple Tg, CD4+Foxp3− TIL were analyzed for binding to a p776/HLA-DR1–specific tetramer (1m) and for...
intracellular IFNγ levels. In this way, we show low levels of p776-binding Th in their TIL in p435-vaccinated neu− littermates (Fig. 4Ba). The majority (90%) of these CD4+Tm+ TIL also expressed IFNγ (Tm+IFNγ+; Fig. 4Ba). The percentages of functionally active CD4+Tm+IFNγ+ Th TIL were highly increased in neu− littermates upon vaccination with the combination vaccine (Fig. 4Bb and Cb). In contrast, very few Th TIL were functionally active (i.e., CD4+Tm+IFNγ+) in tolerant neu+ triple Tg upon vaccination with p435 (Fig. 4Aa). This situation was changed upon immunization with p776 plus p435; the percentage of Th was significantly increased, and importantly the vast majority of CD4+Tm+ cells were also CD4+IFNγ+(Fig. 4Ab and Ca). In contrast to Th TIL, Treg TIL did not seem to be peptide specific, as we could not detect

**Figure 2.** Quantitative and qualitative differences in p435-specific CD8+ effector TIL in neu+ triple Tg versus neu− littermates. Aa and Ba, PENT+ analyses of CD8+ TIL in neu+ triple Tg or neu− littermates vaccinated with p435. Gated CD8+PENT+ cells were analyzed for intracellular IFNγ and CD107a (Aa, Ba). Ab and Bb, flow cytometric analyses for CD8+PENT+ TIL, expressing IFNγ or CD107a, from neu+ or neu− littermates injected with p435 plus p776. C, individually analyzed mice from each group.
CD4⁺Foxp3⁺Tm⁺ cells in vaccinated neu⁺ or neu⁻ animals (Supplementary Fig. S3).

**Tregs are susceptible to FasL-Fas-induced apoptosis.** It has been reported that Tregs, in contrast to Th cells, are susceptible to FasL-mediated cell death (25, 26). In our studies, the vast majority (almost 80%) of Th TIL in neu⁺ triple Tg upregulated FasL (i.e., CD4⁺Foxp3⁻Fas⁺FasL⁺ and CD4⁺Foxp3⁻ Fas⁻FasL⁺) upon vaccination with the peptide combination vaccine (Fig. 5Ab versus Aa and Ac). The peptide combination vaccine did not trigger FasL upregulation in Tregs from the same group of mice (Fig. 5Ab versus Ac). These data suggested that p776-induced FasL⁺Th TIL may act to keep Treg TIL numbers low by modulating their survival via FasL-mediated killing. To test this, we analyzed Treg TIL from neu⁺ triple Tg, treated with our peptide combination vaccine, for apoptosis using Annexin V. A great percentage of apoptosis was apparent in Treg TIL (defined as CD4⁺Foxp3⁺Annexin V⁺ cells) from p435 plus p776–vaccinated neu⁺ triple Tg (Fig. 5Bb and Bc), whereas only low percentages of apoptosis undergoing Treg TIL could be detected after vaccination with p435 alone (Fig. 5Ba and Bb). In contrast to Treg TIL, low numbers of Annexin V⁺ cells could be detected among Th TIL in either group of vaccinated neu⁺ triple Tg (Fig. 5Ba and Bb). Similar results (i.e., increased percentages of FasL⁺ Th and Annexin V⁺ Tregs upon vaccination with the peptide combination vaccine) were also observed in neu⁻ littermates (Supplementary Fig. S4).

Figure 3. p435 plus p776 treatment increases the ratio of Th to Tregs intratumorally and in the spleen. TIL (A–C) or splenocytes (collected 1 and 15 d after vaccination; D) were analyzed by flow cytometry for their content of CD4, Foxp3, and CD25 expression. A and B, representative dot plots for vaccinated neu⁺ or neu⁻ animals. C, Th/Tregs ratios in individually analyzed animals. D, percentage of splenic Tregs.

**A Novel Model for Studying Tolerance to HER-2/neu**
To firmly confirm an interaction between FasL⁺ Th and Fas⁺ Treg TIL, resulting in increased apoptotic rates among the latter cell population, we isolated Th TIL from neuT⁺ triple Tg vaccinated either with the peptide combination vaccine or with p435 alone and incubated Th from each group for 24 hours with Tregs isolated from p435-vaccinated neuT⁺ triple Tg. The data from Fig. 5Cb and Cc show high percentages of Annexin V⁺ Tregs (average, 72%) upon incubation with FasL⁺ Th from p435 plus p776–vaccinated mice. In contrast, only a minor population of Tregs was Annexin V⁺ upon incubation with Th from neuT⁺ mice vaccinated with p435 alone (average, 5%; Fig. 5Ca and Cc).

In another model, we applied intratumoral injections with anti-FasL mAb in p435 plus p776–vaccinated neuT⁺ triple Tg (n = 8) at a time period before tumor shrinkage (i.e., 30–40 days after tumor inoculation). In all these mice, tumor growth was promoted (Fig. 1Dc). In contrast, intratumoral injections with an isotype-matched antibody did not influence tumor regression (compare Fig. 1Dc with Fig. 1Cc).

Therapeutic vaccination against spontaneous mammary tumors. Female neuT⁺ triple Tg have a 100% penetrance of mammary carcinogenesis and follow similar kinetics of autochthonous tumor development as their parental BALB-neuT animals (Fig. 6A). We next evaluated the effect of HER-2/neu vaccination in the prevention of spontaneous mammary tumors in our female neuT⁺ triple Tg. First, we studied the effect of p435 vaccination given to the mice at week 6 from birth when atypical hyperplasia is already

![Figure 4. HER-2 (15776)–specific CD4⁺ Th are functionally inactive in neuT⁺ triple Tg. Dot plots of HLA-DR1/p776 tetramer (Tm⁺) CD4⁺ T cells and of IFNγ⁺CD4⁺ T cells within the CD4⁺Foxp3⁺ Th TIL of vaccinated neuT⁺ triple Tg (A) or neuT⁻ littermates (B). Representative of five mice analyzed per group. C, p776-specific (Tm⁺) IFNγ⁺ CD4⁺ T cells in individually analyzed animals.](image-url)
evident in the mammary glands but before in situ carcinomas are seen (8). The average number of tumors increased equally fast in both vaccinated as well as nonvaccinated control mice: at least one carcinoma (mean diameter, >2 mm) was palpable in each control (IFA+ GM-CSF) or p435+ IFA+ GM-CSF–vaccinated mouse by weeks 18 to 20, whereas by week 30, a tumor was palpable in all 10 mammary glands (Fig. 6B and C). Vaccination with the peptide combination vaccine delayed by almost 15 weeks the time required for these mice to develop tumors, extending both mouse

Figure 5. Increase of apoptotic Treg TIL in neuT+ triple Tg upon coimmunization with p776. A, analyses of expression of Fas and FasL on gated CD4+Foxp3+ (Tregs) and CD4+Foxp3+ Th TIL isolated from neuT+ triple Tg vaccinated as indicated in Aa and Ab. Ac, dot plots are representative from one of five mice analyzed. B, analysis of expression of Annexin V on gated CD4+Foxp3+ and CD4+Foxp3− cells. Bc, dot plots are representative from four experiments performed. Ca and Cb, in vitro incubation of Tregs from p435-vaccinated neuT+ triple Tg or with Th from p435 plus p776–vaccinated neuT+ triple Tg, respectively. Representative dot plots from one of the three experiments performed (Cc) are presented in Ca and Cb. In each experiment, Tregs or Th were isolated from pooled TIL isolated from five mice vaccinated as indicated. Cc, percentage of Annexin V+ cells gated on CD4+Foxp3+ cells and percentage of FasL+ cells gated on CD4+Foxp3− cells.
Importantly, 30% of the animals vaccinated with p435 plus p776 were tumor free (Fig. 6B) at 1 year of age, when the experiment ended.

**Discussion**

In this study, we explored the possibility that p776-activated Th cells assist in breaking tolerance against the HER-2/neu CTL epitope p435, which represents a tumor rejection molecule (13). In our study, neither p435 nor p776 showed antitumor activity when used as single-peptide vaccines in HER-2/neu tolerant male A2.1/DR1 × neuT+ triple Tg with established TUBO.A2 tumors. By contrast, when immunized with the CTL plus Th HER-2/neu peptide combination vaccine, these animals retarded tumor growth or became long-term survivors by converting tolerogenic into immunogenic conditions. Additionally, we analyzed individual tumors from triple Tg belonging to the CTL-vaccinated group and uncured or cured CTL plus Th–vaccinated groups for immune effector capability. The data showed that the regressing tumors were infiltrated by a significantly higher number of vaccine-specific CD8+ CTL with lytic capabilities and IFNγ production. As concerns the intratumoral presence of CD4+ T cells, we also observed an increased expression of p435-specific CD4+ T cells producing IFNγ in the regressing tumors of triple Tg vaccinated with both peptides compared with the single CTL peptide–vaccinated animals. TIL in various human cancers have been shown to be capable of inhibiting tumor growth, and their presence has occasionally been associated with an improved prognosis (27–29). Moreover, in accordance with our data that show that IFNγ-secreting CD4+ T cells infiltrated regressing tumors, recent studies have suggested that concurrent infiltration by Th1 cells have a positive effect on clinical outcome (30, 31). However, very little is known about the factors/mechanisms that drive these T cells to migrate inside a tumor. The IFNγ secretion we detected at the systemic level (Supplementary Fig. S2) could play a determinant role in this process. Indeed, Nakajima and colleagues have reported that, in IFNγ-deficient mice, functional T cells were generated but failed to migrate to tumor sites (32).

Vaccines directed against HER-2/neu have proved to be successful in the prevention of tumor growth in transplantable tumor models as well as in HER-2/neu Tg mice (33–36). The induction of effective HER-2 antitumor immunity seems to rely on different mechanisms, depending on the tumor model, and there is evidence that both antibodies and CTLs play a role in HER-2 tumor protection after DNA vaccination (33, 36). Thus, at this stage, we cannot rule out the possibility that p776-driven Th cells could assist in HER-2/neu–specific antibody production, thereby also contributing to HER-2/neu tumor protection.

When vaccinated but in the absence of the p776 Th peptide, NeuT+ triple Tg contained low, but measurable, frequencies of such p435 peptide–specific CD8+ T effectors, showing an ongoing vaccine-specific immune response even in the context of a strong tolerogenic environment. Notably, our
phenotypic analysis of the CD4+ Th cell population in TIL indicates that there were also differences in the numbers of p776-specific CD4+ Th cells in the tumor microenvironment between neuT− littermates and neuT+ triple Tg vaccinated in the absence of the Th peptide. In these groups of mice, p776-binding Th in their TIL were low and most probably arose by presentation of endogenous p776 peptides released from TUBO.A2 cells, lysed to a certain extent by the p435-specific CTL or by cells belonging to the innate immune system. This may explain the higher frequencies of CD4+Tm+ cells observed in neuT− littermates versus neuT+ triple Tg vaccinated with p435. More importantly, upon treatment with the intact vaccine formulation (i.e., also that includes p776), the frequencies of CD4+Tm+ Th cells were equally augmented in neuT+ littermates (8-fold versus 6-fold, respectively).

Basically, our data are in line with those recently published by Rolla and colleagues (5), who showed that central tolerance in neuT mice acts by silencing the CD8+ T-cell component of the TCR repertoire whereas the CD4+ T-cell repertoire escapes tolerance induction. Our findings on the frequencies of HER-2 peptide–specific CTL and Th cells in neuT+ animals immunized without the Th peptide (Figs. 4 and 5) suggest that, although tolerance contributes to impairment of immune responsiveness, a potentially effective antitumor repertoire is available even in this strongly tolerogenic contest. Promoting the expansion of this repertoire may result in the generation of effective antitumor responses.

One major finding in our study is that the number of local Tregs can be modulated by effector Th cells. It has recently been shown that an increased effector to Treg ratio in the tumor following CTLA4 blockade and GM-CSF combination immunotherapy correlates with improved survival of tumor-bearing mice (23). We have observed a tremendous increase in the percentage of FasL+ cells among the CD4+Foxp3+ tumor-infiltrating Th in neuT+ triple Tg immunized with the peptide combination vaccine, which, upon cell-cell interaction, mediated apoptotic effects in Fas+ Tregs. To this end, it is worth mentioning that the number of Annexin V+ Tregs was always higher than their percentage of Fas expression (Fig. 5). One possible explanation for this could be that, in addition to Fas-FasL, other death receptors (e.g., DR5) may also be triggered by their ligands expressed on Th. Another possibility, which is based on a recent report (37), could be that our vaccine-activated Th may induce (HLA-DR7+) Treg death through production of Granzyme B. These possibilities are now under investigation.

In agreement with our finding, Chen and colleagues (38) have recently shown susceptibility of Tregs to soluble FasL-induced apoptosis. Thus, our data support the idea that a more potent antitumor response at the tumor site can be achieved via a vaccination regimen generating high numbers of FasL-expressing intratumoral CD4+ Th, which keep Tregs in check by modulating their survival via Fas-mediated killing, which in turn results in higher ratios of Th to Tregs. In this way, we provide a simple method for depleting Treg at the tumor site without the need to exogenously administer Treg-modulating agents, which may cause toxic effects (8, 36).

Although the significance of CD4+ T cells in antitumor CTL responses is recognized, only a few mechanisms have been put forward to explain their positive effects on effector function of CTL (12). In this study, we have shown that, in the tumor microenvironment, HER-2/neu peptide–specific Th potentiates HER-2/neu peptide–induced antitumor responses of CTL via mechanisms including increased IFNγ production and elimination of Treg through FasL-Fas interaction. These mechanisms may have direct implications on the outcome of immune responses to tumors after combined immunotherapies. Surely, further work is needed to precisely elucidate the mechanisms by which Th cells enhance the functional program of CTL and to further delineate the physiologic role of this protective effect.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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