IL-2 Immunotoxin Therapy Modulates Tumor-Associated Regulatory T Cells and Leads to Lasting Immune-Mediated Rejection of Breast Cancers in *neu*-Transgenic Mice[1]

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Studies in cancer patients have suggested that breast tumors recruit regulatory T cells (Tregs) into the tumor microenvironment. The extent to which local Tregs suppress antitumor immunity in breast cancer is unknown. We questioned whether inhibiting systemic Tregs with an IL-2 immunotoxin in a model of *neu*-mediated breast cancer, the *neu*-transgenic mouse, could impact disease progression and survival. As in human breast cancer, cancers that develop in these mice attract Tregs into the tumor microenvironment to levels of ~10–25% of the total CD4$^+$ T cells. To examine the role of Tregs in blocking immune-mediated rejection of tumor, we depleted CD4$^+$CD25$^+$ T cells with an IL-2 immunotoxin. The treatment depleted Tregs without concomitant lymphopenia and markedly inhibited tumor growth. Depletion of Tregs resulted in a persistent antitumor response that was maintained over a month after the last treatment. The clinical response was immune-mediated because adoptive transfer of Tregs led to a complete abrogation of the therapeutic effects of immunotoxin treatment. Further, Treg down-modulation was accompanied by increased Ag-specific immunity against the *neu* protein, a self Ag. These results suggest that Tregs play a major role in preventing an effective endogenous immune response against breast cancer and that depletion of Tregs, without any additional immunotherapy, may mediate a significant antitumor response.   *The Journal of Immunology*, 2006, 177: 84–91.

B ased on several observations in recent years, it has been suggested that breast cancer is a naturally immunogenic tumor (1–9). This naturally induced immunity to breast cancer, however, is not sufficient to block tumor growth. Several potential immune evasion mechanisms have been identified including loss of Ag processing and presentation, production of immunosuppressive cytokines, and recruitment of immunosuppressive cells into the microenvironment, such as plasmacytoid dendritic cells, and regulatory T cells (Tregs)[3] (10, 11).

An intense focus on Tregs in recent years has aided in our understanding of how these specialized T cells are able to dampen immunity (12). In breast tumors, it has been observed that Tregs are associated with the tumor microenvironment (13, 14). However, it is unclear whether this association is pathologic. Unlike other tumor types such as fibrosarcoma, the numbers of Tregs that associate with breast tumors are fewer, and in fact represent only a small minority of the total CD4$^+$ T cells (13–16). Thus, methods for selectively depleting Tregs are required to discern their role in tumor immune evasion.

In this study, our goal was to further understand the potential role of breast cancer-associated Tregs in tumor growth and pathogenesis. We used the rat *neu*-transgenic mouse to discern that role (17). In this model, Tregs infiltrate into breast cancer lesions to levels similar to those observed in human breast cancer as a small subset of the total CD4$^+$ T lymphocytes. To selectively deplete the Tregs systemically, we used an IL-2 immunotoxin fusion protein, Denileukin Diftitox (18, 19). This fusion protein consists of the enzymatically active fragment A of diphtheria toxin, the membrane-translocating portion of diphtheria toxin fragment B, and human IL-2. Studies have shown that the immunotoxin binds specifically to IL-2R in vitro and is rapidly internalized via receptor mediated endocytosis (18, 19). Immunotoxin treatment resulted in the selective depletion of Tregs without eliciting additional cytopenia. Furthermore, simple inhibition of Tregs without any additional immunomodulation had a profound effect in inhibiting tumor growth and stimulated an increase in tumor-specific immunity.

Materials and Methods

Animals

BALB/c and *neu*-transgenic mice were obtained from The Jackson Laboratory (17). The *neu*-transgenic mice harbor the nonmutated, nonactivated rat *neu* proto-oncogene under control of the mouse mammary tumor virus promoter. In these studies, only female mice, 8–12 wk old, were used for experimentation. Animal care and use was in accordance with institutional guidelines.

Reagents

FCS was obtained from Gemini Bioproducts. RPMI 1640, PBS, penicillin-streptomycin, and L-glutamine were obtained from Invitrogen Life Technologies. Fluorochrome-conjugated Abs targeting CD3, CD4, CD25, CD62L, CD69, purified and biotinylated anti-IFN-γ, and purified glucocorticoid-induced TNF-related receptor (GITR) Ab were obtained from BD Pharmingen. The IL-2 immunotoxin, Denileukin Diftitox, was a gift from D. Woo and K. Brady of Ligand Pharmaceuticals. Denileukin Diftitox is very rapidly cleared and in humans and rodents,

the drug has a half-life of <2 h in the clearance phase (Ligand Pharmaceuticals, unpublished observation and drug package insert). [^{3}H]Thymidine was purchased from PerkinElmer. The CD4$^+$CD25$^+$ (Treg) T cell purification kit was purchased from Miltenyi Biotec. Alkaline phosphatase-conjugated goat anti-mouse Ig and NBT-chloride/5-bromo-4-chloro-3-indolyl-phosphate color development solution were obtained from Invitrogen Life Technologies. In general, all other reagents were purchased from Sigma-Aldrich. Foxp3 and β-actin primers and TaqMan Universal PCR Master Mix were obtained from Applied Biosystems, and Foxp3 Ab was obtained from eBioscience. Monoclonal mouse anti-CD25 Ab was produced using the PC 61.5.3 hybridoma (American Type Culture Collection (ATCC)). A first-strand cDNA synthesis kit was purchased from Novagen. The H-2Dq/RNEU$_{420-429}$ (H-2D(q)PDSLRDLSVF) tetramer was obtained from the National Institute of Allergy and Infectious Diseases MHC Tetramer Core Facility (Atlanta, GA).

Cell lines

The mouse mammary carcinoma (MMC) cell line was established from a spontaneous tumor harvested from the *neu*-transgenic mice as previously described (20). MMC cells were grown and maintained in RPMI 1640 supplemented with 20% FCS as well as penicillin/streptomycin and L-glutamine.

T cell enrichment

Tumor-infiltrating lymphocytes (TIL) were harvested by mincing the tumor and screening. The TIL were then isolated from tumor cells by centrifugation of the cell suspension on a discontinuous Ficoll gradient consisting of a lower 100% layer and an upper 75% layer. The cells were gently layered onto the top of the gradient followed by centrifugation at 280 × g for 30–45 min at 4°C followed by two washes in HBSS. The TIL were collected at the top of the 75% layer and the tumor cells at the top of the 100% layer. The cells were then stained for flow cytometric analysis as described below. For isolation of CD4$^+$CD25$^+$, the Miltenyi Biotec mouse Treg purification kit was used in conjunction with an AutoMacs machine (Miltenyi Biotec). The resulting CD4$^+$CD25$^+$ T cell population was consistently >95% pure as assessed by flow cytometry. Purified CD4$^+$CD25$^+$ T cells were then used in MLR assays as described below or reinfused into tumor-bearing animals.

Tumor growth in vitro and in vivo

For tumor studies, MMC cells, which are syngeneic with the *neu*-transgenic mouse, were used. For in vitro experiments, $1.0 × 10^5$ MMC were plated in 6- or 96-well plates with medium alone or with varying concentrations of Denileukin Diftitox. For evaluation by flow cytometry, the wells were harvested with a NaCl solution (0.8%) with 2 mM EDTA. Proliferation analysis was done as previously described (21). For in vivo tumor growth, MMC cells were harvested using 2 mM EDTA in PBS and washed before injection. Mice were inoculated with $5 × 10^6$ MMC cells s.c. on the mid-dorsum with a 23-gauge needle, which is a dose of tumor cells that results in the development of tumors in 100% of *neu*-transgenic mice. Tumors were measured every other day with vernier calipers and tumor volume was calculated as the product of length × width × height × 0.5236. In vivo data are presented as mean ± SEM. The numbers of mice used in each experiment are described in *Results*. For in vivo studies, statistical significance ($p < 0.05$) was determined using Student's *t* test by comparing the means of different treatment groups (GraphPad InStat for Windows 95/NT; GraphPad). Mice were treated every 2–3 days with tail vein dosing of either 100 μl of PBS as control or 100 μl of PBS containing varying concentration of the immunotoxin. Mice received either three or six doses of immunotoxin which is specifically stated in the figure legends. In one experiment, a depleting anti-CD25 Ab was used in parallel with the IL-2 immunotoxin for in vivo therapy. In this experiment, mice were treated as previously described with a single 1-mg dose of anti-CD25 Ab given 2 days before tumor cell injection (22).

Flow cytometry

Cell surface and intracellular marker analysis of splenocytes and TILs was done as previously described (20). Samples were run on a FACS Scan II and analyzed using CellQuest software (BD Biosciences). Foxp3-PE intracellular staining was done according to the manufacturer's intracellular staining protocol. For the tetramer experiments, mice were treated with Denileukin Diftitox followed by injection of $5 × 10^6$ irradiated MMC tumor cells 2 days later (>10 drug half-lives). For the Foxp3 intracellular staining, values were considered significantly lower if they were below the

mean and 3 SDs of the control mice value. Means were calculated from appropriate quadrant values and statistical analysis of data was done using Graphpad InStat as described above.

Proliferation assays

In vitro tumor cell and T cell proliferation were examined using a tritiated thymidine incorporation assay in 96-well plates as previously described (23). In brief, for the T cell proliferation assays, the cells were exposed for 5 days to control or experimental compounds or irradiated allogeneic stimulator cells. Allogeneic stimulators were derived from BALB/c mice spleens and irradiated to 3300 rad before use in the MLR. The stimulator cells ($1 × 10^5$) were mixed with splenocytes derived from the *neu*-transgenic mice at a 1:1 ratio. CD4$^+$CD25$^+$ T cells, derived from the *neu*-transgenic mice, were added to a final concentration of either $1 × 10^4$ or $1 × 10^5$ cells. On day 5, 50 μl of medium containing 10 μCi [^{3}H]thymidine was added per well for 8 h followed by harvesting the cells and measuring the uptake of the [^{3}H]thymidine into the DNA of the cells. Data are expressed as the mean percentage of control uptake or as a stimulation index calculated as the ratio of the mean value of the experimental wells over the mean value of the control wells.

Foxp3 mRNA analysis

Normalized PCR analysis of lymphocyte Foxp3 mRNA was done similar to what has been previously described except substituting β-actin as the normalization gene (24, 25). Total RNA was extracted from TIL cells using an RNAqueous-4PCR kit (Ambion). The cDNA synthesis was conducted using a first-strand cDNA synthesis kit. Quantitative real-time PCR was performed on a Prism 7900 sequence detection system using a primer mix for either Foxp3 or β-actin. The reaction mixtures (20 μl of total volume) contained 10 μl of TaqMan Universal PCR Master Mix, 9 μl of diluted cDNA, along with 1 μl of either primer mix. The reactions were amplified as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. Relative Foxp3 mRNA expression in immunotoxin-untreated and treated mice was determined by normalizing to that of β-actin expression in each sample. The expression in immunotoxin-treated mice is presented as fold-change in mRNA values using untreated mice as a reference sample.

ELISA detection of tumor Ag-specific Abs

Serum samples were taken before and after the mice received immunotoxin or control PBS treatment. The sera were used to measure Ab response to a panel of eight SEREX-identified mouse tumor Ags (C3, Eprs, Krt2–8, Lass2, Rock1, Srpk1, Swap70, Yb1) using crude lysate ELISA. XLOLR bacteria were transformed with plasmid encoding the gene of interest or plasmids with no inserts (reference lysate) were grown in Luria-Bertani medium. Protein expression was induced with 2 mM isopropyl β-D-thiogalactoside. After overnight culture at 37°C, the bacteria were spun-down and resuspended in PBS with protease inhibitor (Roche). Cells were then disrupted by freeze-thawing and vortexing. Protein concentration was determined by BCA protein assay kit (Pierce). Bacterial lysate was frozen at −70°C until use. Ninety-six-well Immulon 4HBX microtiter plates (Dynex Technologies) were coated with XLOLR bacteria lysate (50 μg/ml diluted in carbonate buffer). Plates were coated overnight at 4°C using lysate from bacteria that express the protein or reference lysate. After blocking with 100 μl/well of PBS/1% BSA at room temperature on a rocker for 1 h, plates were washed four times with PBS/0.5% Tween 20. After washing, 50 μl of 1/100 diluted mouse sera was added to each well and incubated at room temperature on a rocker for 2 h. After serum incubation, plates were washed four times with PBS/Tween 20 and goat anti-human IgG-HRP conjugate (Zymed Laboratories; 1/5000 diluted) was added and incubated for 1 h at room temperature on a rocker. After a final PBS/Tween 20 wash, TMB developing reagent (Kirkegaard & Perry Laboratories) was added (75 μl/well) and the reaction was then stopped with 75 μl/well 1 N HCl and read at an OD of 450 nm. The OD of each serum sample was calculated as the OD of the Ag lysate-coated wells minus the OD of reference lysate-coated wells. Statistical analysis was performed as described above.

Results

The numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells are markedly increased in the breast tumors of neu-transgenic mice as compared with the peripheral circulation

In mice, GITR, CD62L, CD69, and Foxp3 have been shown to identify Treg populations, distinguishing them from activated

CD4$^+$CD25$^+$ (26, 27). Using a combination of these markers as well as CD25 to define Treg phenotype, we examined the baseline levels of Tregs in the spleen from non-tumor-bearing *neu*-transgenic mice (Figs. 1, *A–E*). CD4 and CD25 distinguished a population that constituted 10.4 ± 1% of spleen CD4 T cells. All four markers, CD62L, CD69, GITR, and Foxp3, identified a subpopulation of CD4$^+$CD25$^+$ T cells. CD62L, a molecule that mediates binding of naive T cells to endothelium, is lost on T cell activation and is expressed constitutively on the Tregs (28). CD62L and CD25 were coexpressed on 6.1 ± 1.1% of spleen-derived CD4$^+$ T cells (*n* = 5, Fig. 1, *A* and *B*). The absence of CD69, an early marker of T cell activation, distinguishes between CD25$^+$ Tregs and activated effector T cells. The CD25$^+$CD69$^-$ T cells, similar to that observed using CD62L as a marker, were 6.1 ± 0.6% of the spleen-derived CD4$^+$ T cells, respectively (Fig. 1, *A* and *C*). GITR also dis-

tinguished a population of similar size (5.8 ± 0.9%) of CD4 T cells compared with CD62L, in the spleen (*n* = 3, Fig. 1, *A* and *D*). Recently, a mAb has become available to demonstrate protein expression of Foxp3, a fairly specific marker for thymic-derived Tregs. Intracellular staining with this Ab revealed that 6.9 ± 0.2% (*n* = 5, Fig. 1, *A* and *E*) of the splenic CD4$^+$ T cells are Foxp3$^+$ which is consistent with the findings using the other markers. Overall, Foxp3 stained 80 ± 2% (*n* = 5) of the CD4$^+$CD25$^+$ T cells suggesting that the vast majority of, but not all, circulating CD4$^+$CD25$^+$ T cells are thymic-derived Tregs (data not shown).

Purified splenic CD4$^+$CD25$^+$ T cells from the *neu*-transgenic mouse could block T cell responses in a MLR assay in a dose-dependent manner (Fig. 1*F*). At a 1:10 Treg:effector T cell ratio, the proliferative response in a MLR was reduced to 67 ± 10% (mean ± SEM, *n* = 4, *p* = 0.07) of the control response, whereas

FIGURE 1. Numbers of CD4$^+$CD25$^+$Foxp3$^+$ T cells are markedly increased in the breast tumors of *neu*-transgenic mice as compared with the peripheral circulation. *A*, Mean numbers of CD4$^+$CD25$^+$ (Total CD25$^+$), CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$CD69$^-$ CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells present in spleen. The data (mean ± SEM) are calculated from 3 to 10 mice each using at least 50,000 gated CD4$^+$ T cells. *B–D*, Representative dot plots for CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$CD69$^-$, CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells, respectively. The values shown represent the percentage that the quadrant represents of the total CD4$^+$ T lymphocytes. *F*, Results of a proliferation assay with values expressed as a mean (±SEM) percentage of the highest proliferation response (S, stimulators; R, responders). Values are representative of three determinations. *G*, The mean numbers (±SEM) of CD4$^+$CD25$^+$CD62L$^+$, CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$Foxp3$^+$ T cells present in the TIL. The data are calculated from 4 to 10 mice each using at least 50,000 gated CD4$^+$ T cells. *H*, The distribution of Foxp3 among the CD4$^+$ T cell population in the tumor bed. The number shown in the *upper right quadrant* is the percentage of CD4$^+$ T cells that stained positive for both CD25 and Foxp3 in this example.
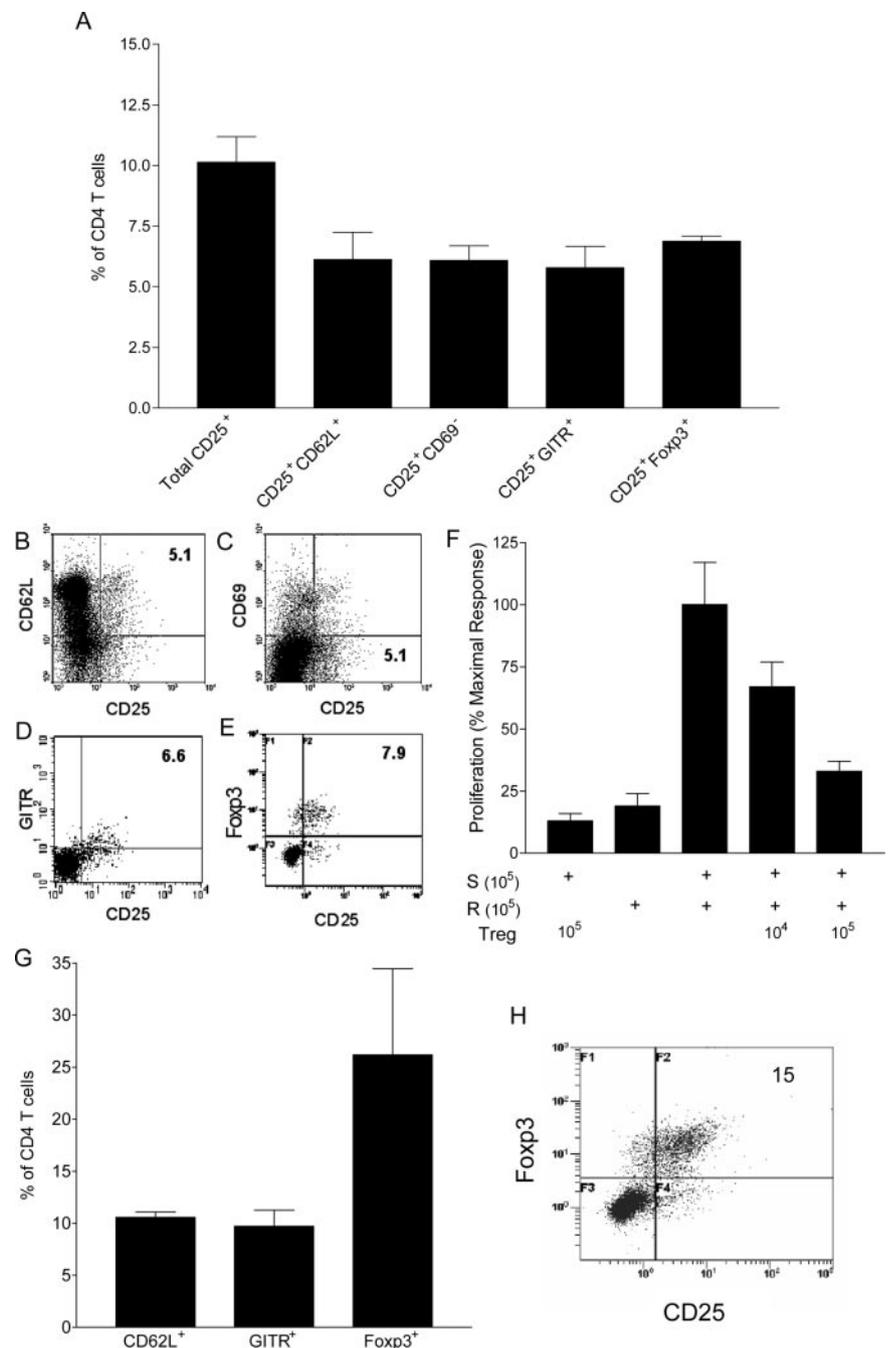
at a 1:1 ratio, the proliferative activity was reduced to 33 ± 4% ($n = 4$, $p = 0.004$) of control response.

An analysis of the TIL revealed that Tregs concentrate in the tumor tissue (Fig. 1, G and H). Tregs, as measured by $CD62L^+$ or $GITR^+$ constituted 10.6 ± 0.5% ($n = 8$, $p < 0.0001$ compared with spleen) and 9.7 ± 1.6% ($n = 9$, $p = 0.016$) of the total $CD4^+$ T cells infiltrating the tumor, respectively. In contrast, Foxp3 (along with CD25) stained 26.2 ± 8% ($n = 5$) of the infiltrating $CD4^+$ T cells suggesting that CD62L and GITR do not represent the entire Treg population or whose levels modulated during activation in the tumor microenvironment. In contrast to studies in humans, we found that levels of Treg markers were not elevated, but slightly decreased, in the periphery following tumor development (e.g., control spleen $GITR^+$ T cells, 6.1 ± 0.6%, $n = 5$; tumor spleen $GITR^+$ T cells, 4.0 ± 0.4, $n = 3$, $p = 0.02$).

CD4⁺CD25⁺Foxp3⁺ T cells can be systemically depleted with an IL-2 immunotoxin without causing profound lymphopenia

In the interest of modulating immunosuppressive T cells, we examined whether $CD4^+CD25^+GITR^+$ T cells, $CD4^+CD25^+CD62L^+$ T cells, or $CD4^+CD25^+Foxp3^+$ T cells could be depleted systemically using the IL-2 immunotoxin, $DAB_{389}IL-2$. Fig. 2A shows that treatment with the immunotoxin can reduce the number of $CD4^+CD25^+GITR^+$ T cells in the spleen. The effects were dose dependent and observed above levels of a 1-μg dose. In animals treated with 5 μg (2.0 ± 0.4, $n = 3$, $p = 0.008$), the levels of Tregs in the spleen were significantly lower than control (PBS-treated) animals (5.8 ± 0.86, $n = 3$). As shown in Fig. 2B, four of six mice demonstrated reduced numbers of $Foxp3^+$ Tregs compared with control levels. During treatment, there was no change in the absolute

number of $CD4^+$ and $CD8^+$ T cells in any of the groups. As shown in Fig. 2C, at the highest dose of immunotoxin, the relative number of CD4 T cells was 68 ± 4% (mean ±SEM, $n = 3$, $p > 0.05$ vs control) of the total spleen cells. This was not significantly different from the control level of 72 ± 0.3%. Similarly, the numbers of $CD8^+$ T cells was also not significantly altered by systemic treatment with immunotoxin. The relative number of splenic $CD8^+$ T cells was 28 ± 2% ($n = 3$) for the 5-μg dose of immunotoxin and was 24 ± 1% ($n = 3$) for the control group ($p > 0.05$).

Mice undergo immune-mediated tumor rejection following IL-2 immunotoxin therapy and demonstrate persistent reductions in tumor-associated Foxp3⁺ T cells

Treatment with immunotoxin was initiated on the day after tumor challenge (i.e., day 1) in *neu*-transgenic mice before tumor development. On day 40, the mean (±SEM) tumor size in animals treated with the 1- and 5-μg dose was 666 ± 94 mm³ ($n = 6$, $p = 0.006$ compared with control day 40) and 310 ± 136 mm³ ($n = 5$, $p = 0.0007$), respectively (Fig. 3A). The mean tumor size of the control mice on day 40 was 1275 ± 156 mm³ ($n = 6$). On day 51, tumors in the mice treated with the 5-μg dose were stable and did not differ in size compared with day 40, despite the fact that the immunotoxin treatment had ended over 30 days before that measurement ($p = 0.5$). Although the tumors in the mice treated with the 1-μg dose did continue to grow, the rate was markedly slower than control animals and on day 51 the mean tumor size was significantly smaller than control (1732 ± 295 vs 3148 ± 360, $p = 0.007$). We evaluated for $Foxp3^+$ T cell mRNA signal in TILs within these tumors using PCR which demonstrated that Tregs were reduced in the tumor microenvironment in the immunotoxin-treated animals (Fig. 3B, $p < 0.03$).

To demonstrate that the effects of the immunotoxin were immunologically mediated, a subsequent experiment was performed to replace Tregs that were removed with immunotoxin. Ten days after groups of tumor-challenged animals were treated with immunotoxin and had demonstrated significant persistent tumor inhibition, we replaced the Treg population either by infusion of unfractionated splenocytes or infusion of $CD4^+CD25^+$ purified T cells. As shown, the Treg infusion led to a complete abrogation of the effects of immunotoxin (Fig. 3C). By day 33, the tumors in the animals treated with immunotoxin were significantly smaller than control (181 ± 29 mm³, $p < 0.0001$). Tumors in the group subsequently infused with purified $CD4^+CD25^+$ T cells (922 ± 219 mm³, $n = 5$, $p = 0.5$) were the same size as tumors in untreated control animals (915 ± 245 mm³, $n = 6$). Splenocytes, also a source of Tregs, partially abrogated the antitumor effect of the immunotoxin when adoptively transferred into immunotoxin-treated animals (436 ± 57 mm³, $n = 6$).

FIGURE 2. Tregs can be systemically depleted without causing profound lymphopenia. *A*, The levels of $CD4^+CD25^+GITR^+$ T cells in the spleen after systemic treatment with varying doses (total of six doses of 5, 2.5, or 1 μg every 2–3 days), shown on the *x*-axis, of immunotoxin or control medium. *B*, Levels of $CD4^+CD25^+Foxp3^+$ T cells in immunotoxin-treated mice, given 5 μg of immunotoxin every 2–3 days for a total of six doses. The levels in *A* and *B* are expressed as a percentage of the total $CD4^+$ T cells. *C* and *D*, The levels of splenic $CD4^+$ and $CD8^+$ T cells, respectively, expressed as the percent of total splenic-derived cells. Each bar represents the mean and SEM of three replicates at each dose.

MMC tumor cells are not directly sensitive to IL-2 immunotoxin

We next considered the possibility that IL-2 immunotoxin treatment may have a direct effect on tumor either by coating the surface for immune activation or by internalizing it to inhibit cell proliferation. As shown in Fig. 4A, the immunotoxin had no impact on tumor cell growth as assessed by an estimate of proliferation. At the highest in vitro dose examined, the proliferative stimulation index was 1.3 ± 0.2 (mean SI ± SEM, $n = 3$), which was not significantly different from medium control (1.0 ± 0.3) or protein (500 ng/ml OVA, oval, 1.0 ± 0.1) control ($p > 0.05$). Further, the tumors themselves do not express CD25 on the cell surface, further explaining the insensitivity to the drug (Fig. 4B).
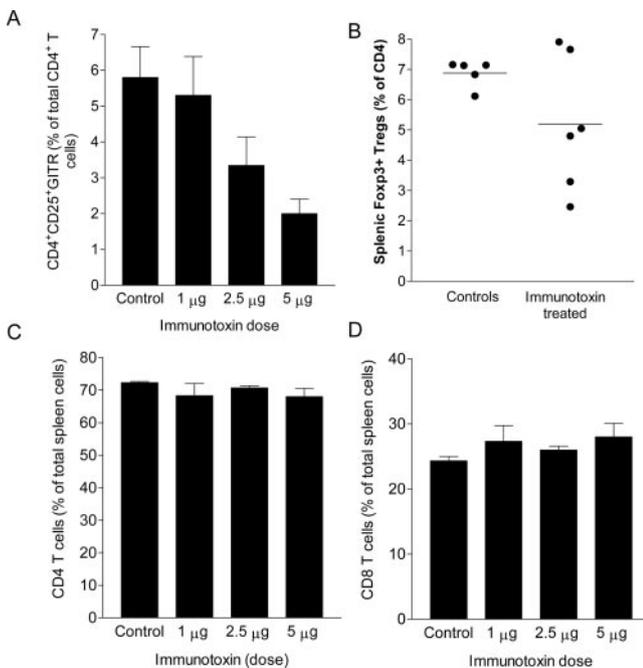
FIGURE 3. Mice undergo immune-mediated tumor rejection following IL-2 immunotoxin therapy and demonstrate persistent reductions in tumor-associated Foxp3$^+$ T cells. *A*, The line spanning the first 17 days of the time course (arrow) represents the immunotoxin (■, 1 μg; ♦, 5 μg) or PBS (control, ●) treatment time points. Mice were given six doses (1 or 5 μg/dose) of IL-2 immunotoxin, one dose given every 2–3 days. Each data point represents the mean tumor size ($\pm$SEM) of five to six mice given in cubic millimeters. Another experiment with six mice yielded similar results. *B*, Levels of intratumoral Foxp3 mRNA (calculated from five to six mice per group) in mouse tumors treated with 5 μg of IL-2 immunotoxin three times every 2–3 days. *C*, The line spanning the first 4 days of the time course (arrow) represents the immunotoxin or PBS (control, ●) treatment time points. Mice were treated with three doses of 5 μg of IL-2 immunotoxin given every 2–3 days. At day 21, a group of immunotoxin-treated mice received a CD4$^+$CD25$^+$ T cells (◇), splenocyte (□) or PBS infusion (■). Each data point represents the mean tumor size ($\pm$SEM) of five to six mice given in cubic millimeters. The closed circles and open diamonds coincide on the graph.
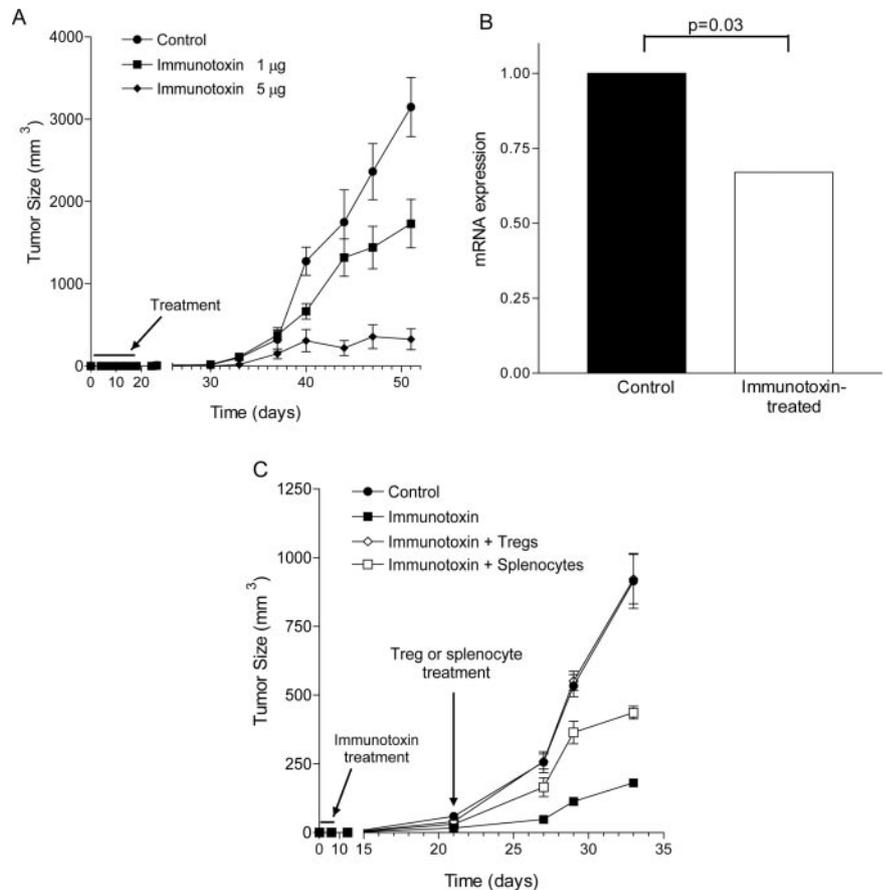
Mice undergoing tumor rejection following Treg depletion with IL-2 immunotoxin demonstrate elevated levels of tumor Ag-specific Abs

Given that the immunotoxin is associated with the down-regulation of Treg and generates an antitumor response, we next questioned whether immunity to the tumor Ags was being induced during treatment. As shown in Fig. 5, immunotoxin treatment was associated with the development of tumor Ag-specific immune responses as assessed by Ag-specific ELISA. Blood drawn from mice ($n = 8$) before and after treatment with the immunotoxin or PBS revealed that immunotoxin-treated mice developed IgG Ab immunity to srpk1, a novel tumor Ag our group has recently identified as a natural immune target in *neu*-transgenic mice (29).

Depletion of Tregs increases the capability of the mice to overcome tolerance to tumor-associated Ag neu

The *neu*-transgenic mouse is highly tolerant to *neu* and therefore we next examined whether immunotoxin treatment elicited a T cell response against the immunodominant epitope of rat *neu*, peptide 420–429, that has been previously reported to be targeted following depletion of Tregs with anti-CD25 mAb (22, 30). As shown in Fig. 6, the depletion of Tregs with immunotoxin led to an increased capability of mice to generate *neu*-specific CD8 T cell immunity as directly assessed with tetramer analysis. Although the results suggest that IL-2 immunotoxin is better than anti-CD25 treatment, the results were not significantly different ($p > 0.05$), which may have been due to the different dosing strategies.

Discussion

Breast cancer is a naturally immunogenic tumor. Several studies have demonstrated that breast cancer lesions are extensively infil-

trated with immune effector cells such as T cells, B cells, and dendritic cells (1, 2, 31–36). Investigators have also observed that a lack of T cell infiltration in breast cancer may be associated with increased invasion of local lymph nodes by disease, indicating that an active immune response can limit the growth and spread of the tumor (35). Furthermore, tumor-Ag-specific immunity can be detected in the blood and bone marrow of breast cancer patients, but not in non-cancer-bearing donors, indicating that exposure to tumor induces a tumor Ag-specific immune response (8, 37, 38). Despite the fact that patients with breast cancer can demonstrate an immune response to their tumors and that T cells can localize to the breast tumor microenvironment, cancer growth remains unchecked. Such observations suggest that there must be a number of mechanisms in the tumor microenvironment that act simultaneously to prevent tumor destruction by the immune system. Our studies suggest that Tregs concentrate naturally in the tumors of *neu*-transgenic mice at levels above that observed in the systemic circulation. Furthermore, we also show IL-2R-directed immunotoxin therapy can reduce systemic levels of CD4$^+$CD25$^+$Foxp3$^+$ Tregs and lead to an increased capability of mice at high risk for tumor development to immunologically reject their tumors for a sustained period of time, even in the absence of therapy. This increased capability of tumor rejection was accompanied by increased tumor Ag-specific immunity.

The *neu*-transgenic mouse demonstrates profound tolerance to the spontaneous breast tumors that develop late in life and the *neu*-specific T cells that can be isolated in these animals are of low avidity (39, 40). Despite the low-avidity immune response, however, the tumor-Ag-specific T cells that remain in the body can demonstrate antitumor activity suggesting that peripheral mechanisms regulate their effector function (40). Presumably, at sites

FIGURE 5. Mice undergoing tumor rejection following IL-2 immunotoxin demonstrate elevated levels of tumor Ag-specific Abs. Shown are the OD450 of ELISA specific for the tumor Ag, srpk1. The OD was calculated as the OD of Ag-coated wells minus the OD of reference lysate-coated wells. Each data symbol represents the Ab level in one mouse serum sample. The data is representative of two independent experiments.
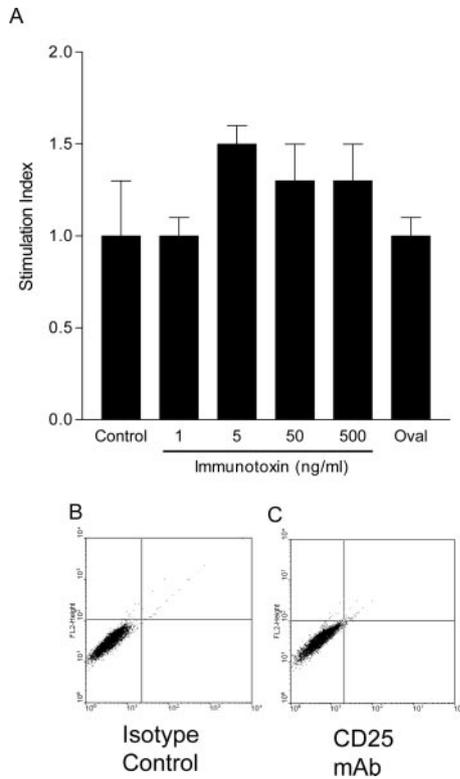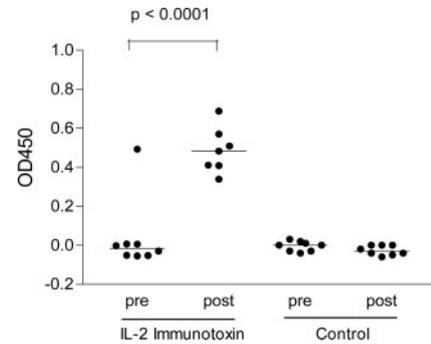
FIGURE 4. MMC tumor cells are not directly sensitive to IL-2 immunotoxin. *A*, Proliferation of tumor cells after exposure to the IL-2 immunotoxin. The results are shown as the mean stimulation index calculated from thymidine incorporation following the exposure of tumor cells to doses of PBS, immunotoxin, or OVA. Each bar represents the mean ($\pm$SEM, $n = 6$ replicates) proliferation expressed as a stimulation index calculated from the control cells. The graph is representative of two independent experiments that yielded similar results. *B* and *C*, Flow cytometric dot plots from two different tumor samples either stained with isotype control Ab (*C*) or stained for CD25. The histograms are representative of two independent experiments that yielded similar results.

where the Ag levels are elevated, such as in the tumor microenvironment, peripheral tolerizing mechanisms must be enhanced, relative to other tissues, to limit effector T cell function specific for self-Ags (41). Data presented here suggests that Tregs are more abundant in the tumors of these mice than in the peripheral circulation, suggesting that they are actively recruited to block effector T cell function and maintain tolerance of Ag-specific T and B cells. Similar to *neu*-transgenic mice, Tregs may be recruited into human breast tumors. Indeed, it has been observed that immunosuppressive $CD4^+CD25^+$ T cells can comprise up to 20% of the total TIL population in humans (13). In vitro studies suggest that Tregs can inhibit Ag-specific T cell activation at levels as low as 10% of the infiltrating T cell population, suggesting that the levels we observed in the tumors are sufficient to block immunity (42, 43). How breast cancers attract Tregs specifically into the microenvironment remains unknown. One recent study in ovarian cancer suggests that the attraction of $CD4^+CD25^+Foxp3^+$ T cells into the ovarian tumor microenvironment is mediated by the chemokine CCL22 which is aberrantly produced by the ovarian tumors (44). Our results suggest that the reduction of Tregs early in the course of disease development prevents the emerging tumors from using Tregs to maintain tolerance. Our finding that the small tumors that do develop in immunotoxin-treated animals have less intratumoral Treg markers (Foxp3) is consistent with this hypothesis.

Given the emerging consensus that Tregs are involved in regulating Ag-specific immune responses in several disease settings such as tumor immunity, autoimmunity, and chronic infections, there is interest in developing Treg-selective depletion strategies for research and therapeutic purposes. One of the problems with defining the role of Tregs is that commonly used strategies such as chemotherapy, anti-CD3, anti-CTLA-4, and anti-CD4 treatment, can result in severe myeloablation or immune dysregulation that could lead to significant toxicities or interfere with generation of immune responses (45–47). Lymphodepletion can suppress immune responses by a number of mechanisms including retarding dendritic cells and macrophages for extended periods, both of which would be required to activate Ag-specific T cells (48). The immunotoxin strategy presented here depleted Tregs without causing myeloablation and resulted in a tumor-specific immune response. The clinical translation of Denileukin Diftitox to deplete Tregs in cancer patients is ongoing. Vieweg and colleagues (49) reported that treatment of cancer patients with Denileukin Diftitox before vaccine therapy could substantially reduce the number of $CD4^+CD25^{high}$ Tregs without altering the levels of other CD4 T cells (memory and naive). Furthermore, this selective depletion led to an apparent enhancement of immunity after vaccination. A recent report evaluated the IL-2 immunotoxin approach as monotherapy for the treatment of advanced stage refractory melanoma (50). The use of Denileukin Diftitox resulted in depletion of $Foxp3^+$ Tregs in some patients but not in others and depletion appeared to be related to dose as we have shown here. Little clinical activity was observed in these patients, which may be due to the advanced stage and potential immunosuppression in these patients. Data presented here used Denileukin Diftitox early in the course of tumor implant, mimicking a more limited stage of disease.

It should be noted that tumors of different tissue origins may use different tolerizing mechanisms. For example, in mouse fibrosarcomas, $CD4^+CD25^+$ T cells constitute nearly 70% of the total lymphocyte (total CD4 and CD8) population and the only effector T cell required for rejection was the $CD8^+$ T cells (16). In our study, we find that $CD4^+CD25^+$ T cells constitute only a minor fraction of the infiltrating CD4 T lymphocytes and this holds true even in more advanced tumors (data not shown). Furthermore, it has been shown that $CD4^+$ Th cells are essential for tumor rejection in *neu*-transgenic mice (15). Investigators demonstrated that depletion of $CD4^+$ T cells diminished the tumor rejection response as well as tumor-specific Ab production. The reconstitution, however, of tumor-specific IgG was able to recover some antitumor
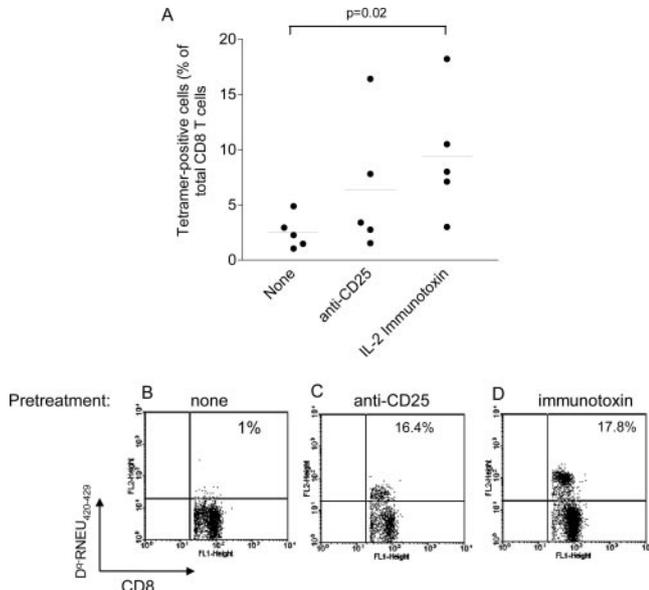
FIGURE 6. Depletion of Tregs increases the capability of the mice to overcome tolerance to tumor-associated Ag *neu. A,* The frequencies of *neu* peptide-specific CD8 T cells as assessed by tetramer analysis in mice treated with PBS, anti-CD25 mAb (1 mg mAb in a single dose), or IL-2 immunotoxin (three doses, 5 μg/dose, 2–3 days apart) 2 days before tumor cell injection. Cells were gated on CD8$^+$CD62L^{low} T cells. *B–D,* Representative dot plots.

responses indicating the important role of the CD4$^+$ T cell in enhancing the Ab responses associated with tumor rejection. Tolerization and antitumor effector mechanisms may be different in different tumors. Data presented here show that inhibition of Tregs both increases the number of tumor-specific T cells as well as allows the generation of tumor-specific Ab immunity. Whether depletion of Tregs would have such a profound effect in other models is not known.

In conclusion, our results strongly suggest that tumors that develop in *neu*-transgenic mice actively recruit Tregs to block tumor rejection and play an important role in suppressing the endogenous immune response to breast cancer. Indeed, simple inhibition of Treg function can lead to an effective antitumor response without any additional immunomodulation. Depleting Tregs in human breast cancer may stabilize disease for extended periods of time or if used in the minimal disease setting may increase the relapse-free period. Understanding the mechanisms by which breast tumors evade immunity could ultimately lead to the better design of immune-based strategies for the treatment of the disease.

Acknowledgments

Disclosures

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