

# Combination of Tumor Site–Located CTL-Associated Antigen-4 Blockade and Systemic Regulatory T-Cell Depletion Induces Tumor-Destructive Immune Responses

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## Abstract

**Accumulating data indicate that tumor-infiltrating regulatory T cells (Treg) are present in human tumors and locally suppress antitumor immune cells. In this study, we found an increased Treg/CD8 ratio in human breast and cervical cancers. A similar intratumoral lymphocyte pattern was observed in a mouse model for cervical cancer (TC-1 cells). In this model, systemic Treg depletion was inefficient in controlling tumor growth. Furthermore, systemic CTL-associated antigen-4 (CTLA-4) blockade, an approach that can induce tumor immunity in other tumor models, did not result in TC-1 tumor regression but led to spontaneous development of autoimmune hepatitis. We hypothesized that continuous expression of an anti-CTLA-4 antibody localized to the tumor site could overcome Treg-mediated immunosuppression and locally activate tumor-reactive CD8<sup>+</sup> cells, without induction of autoimmunity. To test this hypothesis, we created TC-1 cells that secrete a functional anti-CTLA-4 antibody (TC-1/αCTLA-4-γ1 cells). When injected into immunocompetent mice, the growth of TC-1/αCTLA-4-γ1 tumors was delayed compared with control TC-1 cells and accompanied by a reversion of the intratumoral Treg/CD8 ratio due to an increase in tumor-infiltrating IFNγ-producing CD8<sup>+</sup> cells. When local anti-CTLA-4 antibody production was combined with Treg inhibition, permanent TC-1 tumor regression and immunity was induced. Importantly, no signs of autoimmunity were detected in mice that received local CTLA-4 blockade alone or in combination with Treg depletion. [Cancer Res 2007;67(12):5929–39]**

## Introduction

Most tumor-associated antigens (TAA) are nonmutated self-antigens, which are overexpressed or reexpressed in cancer cells. TAA-specific T cells therefore not only react with cancer cells but also have the potential to cause autoimmune reactions. A central problem in cancer immunotherapy is to find approaches that

stimulate antitumor responses without inducing autoimmunity. Regulatory T cells (Treg) are key components for regulating both antitumor immunity and maintaining peripheral immune tolerance toward self (for review, see ref. 1). There is an accumulating evidence that tumor-infiltrating Tregs are present in many human tumors, including ovarian cancer, lung cancer, malignant melanomas, Hodgkin's lymphoma, breast cancer (2–6), and cervical cancer (this study). Human tumors are thought to accumulate Tregs by several mechanisms, including recruitment of peripheral Tregs by intratumorally produced chemokines (2), or conversion of CD4<sup>+</sup> T effector cells to Treg cells by dysfunctional/immature dendritic cells at the tumor site (7). Tregs extracted from tumors inhibit cytotoxicity of autologous CD8<sup>+</sup> T cells *in vitro* and therefore are likely to have a function in suppression of antitumor immune responses *in vivo* (2–6). On the other hand, several reports show a role of Tregs in suppressing autoimmune reactions. Autoreactive T cells seem to be inhibited by Tregs due to cell-cell contact-dependent mechanisms and local secretion of immunoinhibitory cytokines (8). Because of this dual role of Tregs, approaches that involve Treg depletion to enhance antitumor T-cell responses bear the risk of inducing autoimmunity. Several approaches have been used to deplete or inactivate Tregs using antibodies against Treg surface proteins, including the interleukin-2 receptor (CD25; ref. 9), glucocorticoid-induced tumor necrosis factor–related protein (GITR; refs. 10, 11), or CTL-associated antigen-4 (CTLA-4; refs. 12–14). These approaches triggered T effector immune responses toward a spectrum of various cancers but also initiated T-cell-mediated autoimmune diseases, such as gastritis, thyroiditis, sialadenitis, and neuropathy, in several animal models (1, 11, 15, 16). Another approach to delete Tregs involves cyclophosphamide. At low doses (in mice, 30–200 mg/kg; in human, 300–350 mg/m<sup>2</sup>), cyclophosphamide treatment enhances immune responses against a variety of antigens, including tumor antigens, a property that was attributed to the ability of cyclophosphamide to selectively kill Tregs in mice and humans while not decreasing the function of T effector cells (17–20). The mechanism of the selective action of cyclophosphamide on Tregs is currently a central topic of investigation. Recent reports argue that low-dose cyclophosphamide decreases either the number and/or function of Tregs (17–19). In addition to Treg depletion/inhibition, low-dose cyclophosphamide was shown in animal models to induce a Th2/Th1 shift in the cytokine profile, which is thought to further contribute to T-cell-mediated antitumor immune responses (21).

Another approach to overcome Treg-mediated suppression of effector T cells involves manipulation of CTLA-4 signaling. Blockade

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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of CTLA-4 signaling using systemic anti-CTLA-4 antibody administration enhanced tumor immunity in mice (22–24) but also exacerbated/induced autoimmune diseases (25–27). These findings are corroborated by clinical trials, revealing cancer regression and severe autoimmune manifestations in patients with metastatic melanoma on systemic anti-CTLA-4 antibody treatment (28). Two different mechanisms of CTLA-4-mediated suppression of autoreactive cells have been proposed. (a) Transient CTLA-4 expression can be found on activated CD4<sup>+</sup> and CD8<sup>+</sup> T effector cells. CTLA-4 shares its ligands (CD80 and CD86) with CD28 (but has a higher affinity toward them than CD28). On binding to its ligands, CTLA-4 signaling increases the threshold for T-cell activation and thereby inhibits the responsiveness of autoreactive T effector cells (for review, see ref. 29). (b) CTLA-4 is constitutively expressed by Tregs and is thought to be a key molecule for Treg-mediated suppression of self-reactive T cells (6, 12, 14). However, Tregs from CTLA-4<sup>-/-</sup> mice seem capable of *in vitro* suppression (30). Thus, controversy remains about the involvement of CTLA-4 in the suppressive activity of Tregs.

The goal of this study was to induce an effective antitumor immune response without autoimmune side effects by activation of CD8<sup>+</sup> T cells through CTLA-4 blockage and depletion of Tregs. We hypothesized that in contrast to systemic application, tumor-localized expression of anti-CTLA-4 antibodies would confer activation of only tumor-infiltrating T cells, thus reducing the risk of autoimmune reactions. We further speculated that tumor-localized anti-CTLA-4 expression might not be sufficient to suppress Tregs, which are recruited into the tumor from the peripheral blood, and that additional approaches might be necessary to deplete Tregs systemically. To do this, we used treatment with low-dose cyclophosphamide or anti-CD25 antibody in combination with tumor-localized anti-CTLA-4 expression. As a murine cervical cancer model, we used human papillomavirus (HPV)-16 E6/E7-expressing TC-1 tumors, which are infiltrated by both Tregs and CD8<sup>+</sup> T cells. We show that a combination of local anti-CTLA-4 antibody production with systemic Treg depletion enhanced antitumor immune responses and led to effective eradication of TC-1 tumors and induction of antitumor immunity, although no signs of autoimmunity were observed.

## Materials and Methods

**Cells.** TC-1 cells were from the American Type Culture Collection (ATCC) and maintained in RPMI 1640 supplemented with 10% FCS, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Monoclonal rat anti-mouse CD25 antibody (PC61.5 hybridoma, ATCC) and monoclonal hamster anti-mouse CTLA-4 antibody (UC10-4F10-11, clone HB-304, ATCC) were produced by culturing hybridoma cell lines in CELLine 1000 culture flasks (BD Biosciences) using DMEM supplemented with 10% low IgG fetal bovine serum (FBS; Hyclone), 4 mmol/L L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Antibodies were purified from hybridoma supernatant using protein G purification (Amersham Biosciences) followed by dialysis against PBS.

**Generation of TC-1 cell derivatives.** Pseudotyped retrovirus particles were produced by cotransfection of retroviral vectors (pLHCX-αCTLA-4, pLHCX-αCTLA-4-γ1, and pLHCX-αCTLA-4-myl1; see Supplementary Data for construction details) with pVSVG (BD Biosciences) in GP2-293 cells (BD Biosciences). Two days after transfection, the culture medium was filtered, mixed with 8 µg/mL polybrene, and added to TC-1 cells. The cells were selected in hygromycin for 10 days. Expression of recombinant antibodies was analyzed by ELISA (see Supplementary Data).

**Immunohistochemistry.** Analyses of paraffin sections of cervical and breast cancer tissues and mouse organs are described in the Supplemen-

tary Data. Analysis of TC-1 tumor cryosections was done as described elsewhere (31).

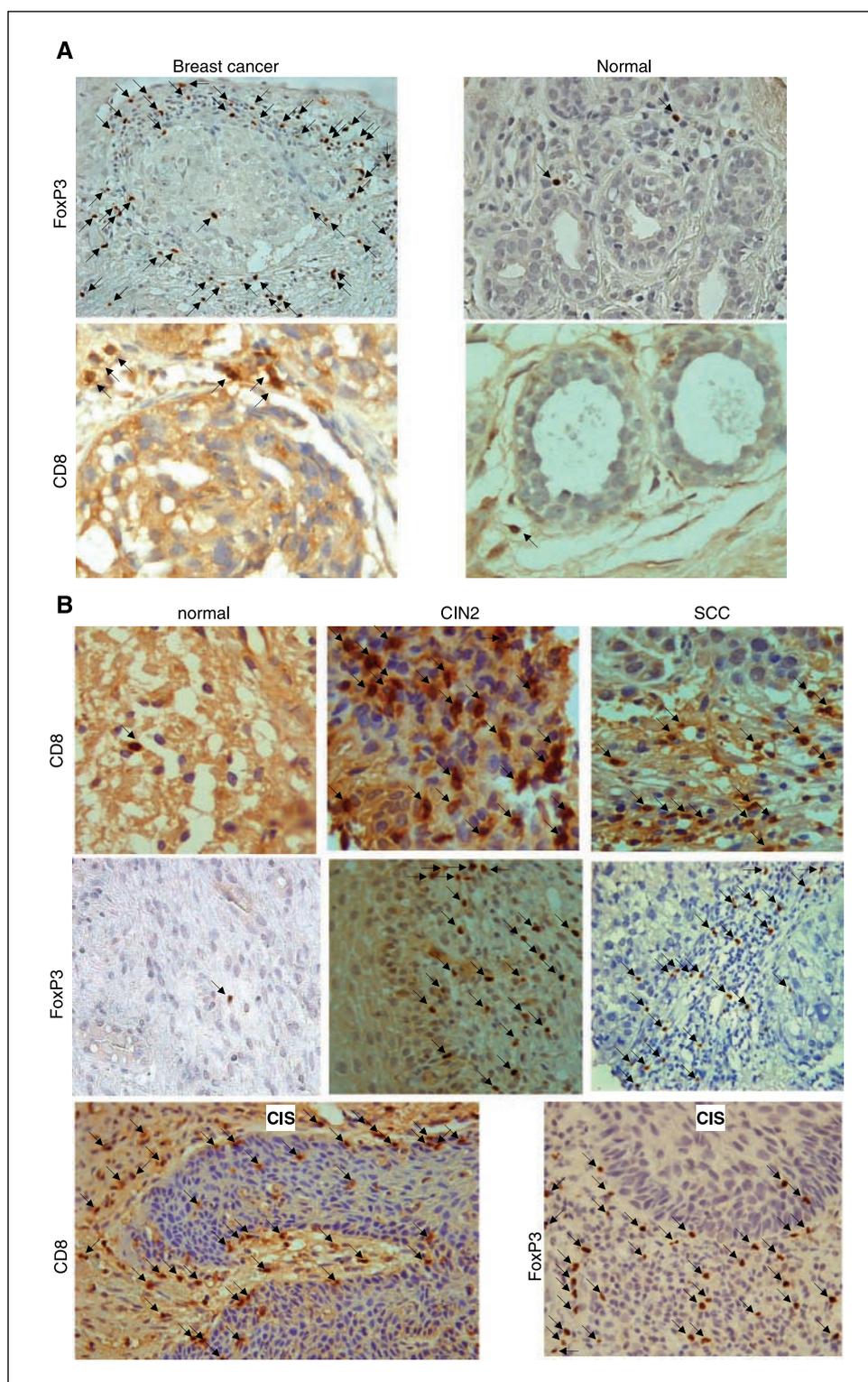
**T-cell enrichment.** Splensens and tumors were minced and filtered through a 70-µm cell strainer (BD Biosciences). The splenocytes and tumor-infiltrating lymphocytes (TIL) were then isolated from tumor cells/erythrocytes by centrifugation of the cell suspension on a Ficoll gradient (Histopaque 1083, Sigma). The cells were layered onto the top of the gradient in a 10-mL Falcon tube followed by centrifugation at 800 × g for 20 min at room temperature without braking. Lymphocytes were collected, washed twice in PBS-1% FBS (washing buffer), and stained for flow cytometric analysis as described below.

**Flow cytometry.** The following monoclonal antibodies (mAb) were used for flow cytometry (final concentration, 5 µg/mL): anti-FoxP3-PE (clone FHK16s, eBiosciences), anti-CD4-PE, anti-CD8-PE, anti-CD8-FITC, anti-CD11b-PE, anti-CD19-PE, anti-CD25-FITC (clone 7D4, all from BD Biosciences), and anti-CD25-FITC (clone PC61.5, eBiosciences; see Supplementary Data for details). For intracellular IFNγ staining, cells were permeabilized, fixed using the Golgi-Plug kit (BD Biosciences), and incubated with anti-CD8-FITC, anti-IFNγ-PE, or anti-CD8-FITC + anti-IFNγ-PE antibodies according to the manufacturer's intracellular staining protocol (BD Biosciences). All samples were treated with Fc-block (anti-CD16/CD32, BD Biosciences). Corresponding isotope controls yielded no significant staining.

**Animal experiments.** All experiments involving animals were conducted in accordance with the institutional guidelines set forth by the University of Washington and the Institute of Biomedical Sciences, Academia Sinica. All mice were housed in specific pathogen-free facilities. Immunocompetent mice (The Jackson Laboratory), severe combined immunodeficient mice (SCID), and CD8 knockout (KO; B6.129S2-Cd8a<sup>tm1Mak</sup>) mice (all C57Bl/6 background) were used. Anti-CD25 antibody, anti-CTLA-4 antibody (hybridomas; see above), control rat antibody (rat IgG), and control hamster antibody (hamster IgG; Jackson ImmunoResearch Laboratories) and cyclophosphamide (Sigma) were i.p. injected in 200 µL PBS. For i.t. injection, 200 µg anti-CTLA-4 antibody or hamster control antibody was injected in 50 µL PBS when TC-1 tumors reached the size of 64 mm<sup>3</sup>. The tumor volume was calculated using the formula [largest diameter × (smallest diameter)<sup>2</sup>]. Mice were sacrificed when the tumor volume reached 1,000 mm<sup>3</sup>.

## Results

**Increased numbers of tumor-infiltrating Tregs and CD8<sup>+</sup> T cells in human breast and cervical cancer patients.** To corroborate published studies on tumor-infiltrating Tregs and the clinical relevance of our mouse model, we studied Treg and effector T-cell numbers in human tumors. In a first study, we analyzed tissue sections of 18 Senegalese stage III and IV breast cancer patients and 5 patients without breast cancer. TILs were stained for nuclear FoxP3 expression as a marker for Tregs. Effector CD8<sup>+</sup> T cells were detected using CD8 as a surface marker (Fig. 1A; Supplementary Table S1). Normal breast tissue had no or only a few FoxP3-positive cells. Cancer tissue showed varying numbers of Tregs (range, 2.2–180/mm<sup>2</sup>; Supplementary Table S1). Compared with normal breast tissue, levels of Tregs were higher in 15 of 17 evaluated cancer patients ( $P < 0.006$ ). CD8<sup>+</sup> lymphocytes (range, 0–100/mm<sup>2</sup>) were elevated in 12 of 17 evaluated patients compared with normal breast tissue ( $P < 0.009$ ). In normal breast tissue, lymphocytes were mostly located near the ducts and glandular tissues. In tumor sections, both Tregs and CD8<sup>+</sup> T cells were predominantly found in the tumor stroma and, occasionally, in tumor nests. Postmenopausal patients showed a higher frequency of both tumor-infiltrating Tregs and CD8<sup>+</sup> T cells, when compared with premenopausal patients, but these differences were not statistically significant (premenopausal versus postmenopausal: FoxP3<sup>+</sup>,  $P < 0.25$ ; CD8<sup>+</sup>,  $P < 0.45$ ). When the numbers of Tregs and CD8<sup>+</sup> T cells were compared in the individual patients, 9 of 12



**Figure 1.** Presence of Tregs and CD8<sup>+</sup> T cells in breast and cervical tissue samples. **A**, Tregs and CD8<sup>+</sup> cells were detected on paraffin sections of normal breast and breast cancer (stage III and IV) tissue. FoxP3-positive cells appear with brown nuclei. CD8-positive cells show a brown surface staining. Representative pictures. *Arrows*, positive cells. **B**, Tregs and CD8<sup>+</sup> cells were visualized on paraffin sections of normal cervix, precancerous cervix, and cervix cancer tissues. FoxP3-positive cells have brown nuclei. CD8-positive cells display a brown surface staining. Representative pictures. *Arrows*, positive cells.

evaluable patients had FoxP3<sup>+</sup> to CD8<sup>+</sup> cell ratios of >1.0 (Supplementary Table S1).

In a second study, we investigated Treg and CD8<sup>+</sup> T-cell levels during cervical cancer progression. We obtained sections of 4 normal cervix tissues, 17 low-grade to high-grade dysplasias [6 cervical intraepithelial neoplasia (CIN) 1, 6 CIN2, and 5 carcinoma *in situ* (CIS)], and 5 invasive squamous cervical cancers (SCC).

Samples were stained for nuclear FoxP3 and CD8 surface expression (Fig. 1B; Supplementary Table S2). Similarly to breast tissue, few FoxP3<sup>+</sup> or CD8<sup>+</sup> cells were found in normal cervix tissue. As with breast cancer, lymphocytes were mostly located in the tumor stroma and less frequently in tumor nests. Significantly higher FoxP3<sup>+</sup> cell numbers were found in dysplasia and cancer tissues than in normal cervix ( $P < 0.0004$ ; Fig. 1B; Supplementary

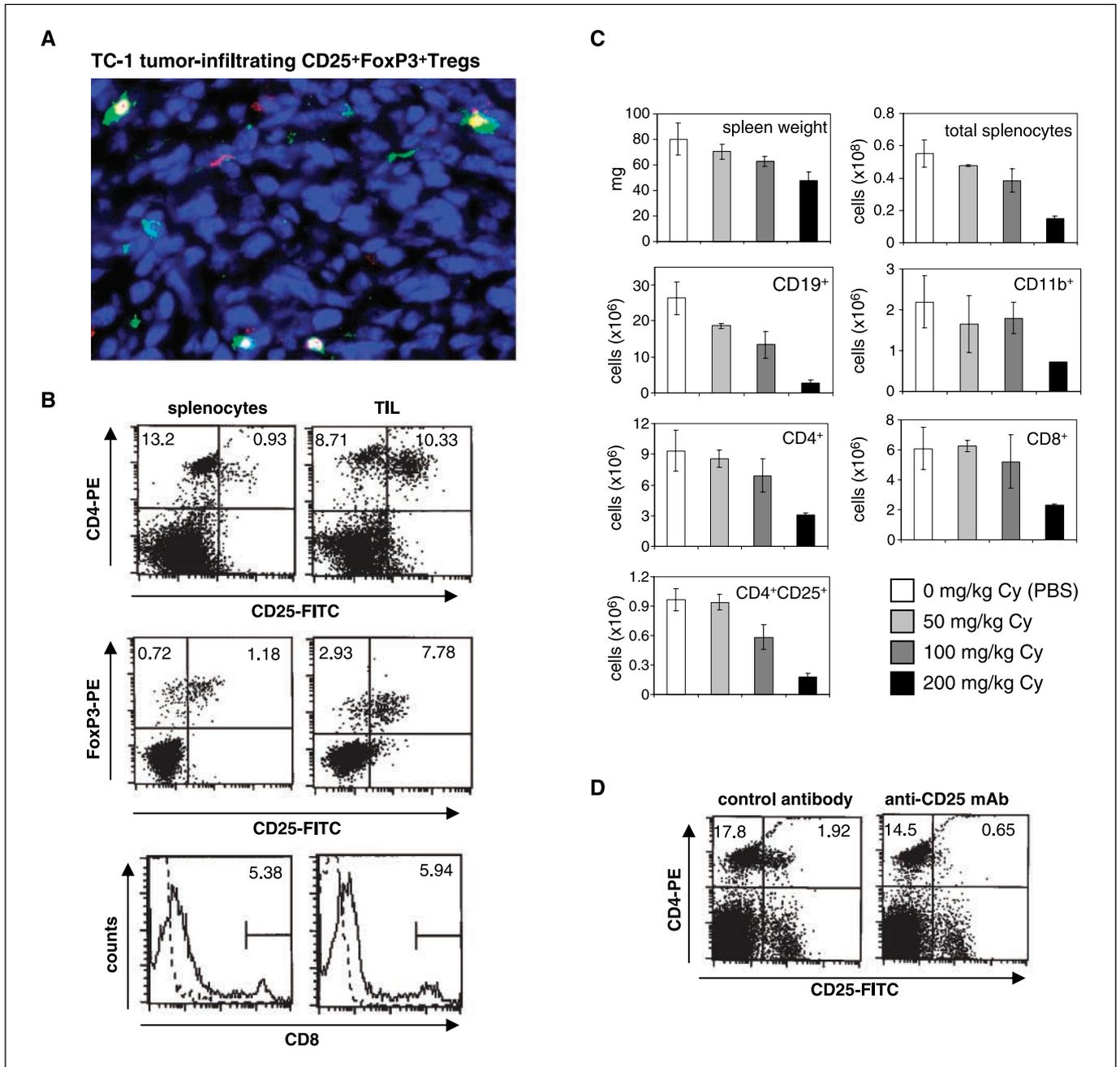
Table S2). CD8<sup>+</sup> counts were significantly higher in premalignant lesions (CIN1, CIN2, and CIS) than in normal cervix ( $P < 0.005$ ). Interestingly, with progression to SCC, CD8<sup>+</sup> cell numbers declined (CIN1, CIN2, and CIS versus SCC;  $P < 0.006$ ).

Taken together, the number of both Tregs and CD8<sup>+</sup> T cells showed a high variability in breast and cervical cancer. Overall,

both lymphocyte fractions were found at higher densities at the tumor site, predominantly in the stroma surrounding the tumor nests.

**Tumor-infiltrating Tregs and CD8<sup>+</sup> T cells in TC-1 tumors.**

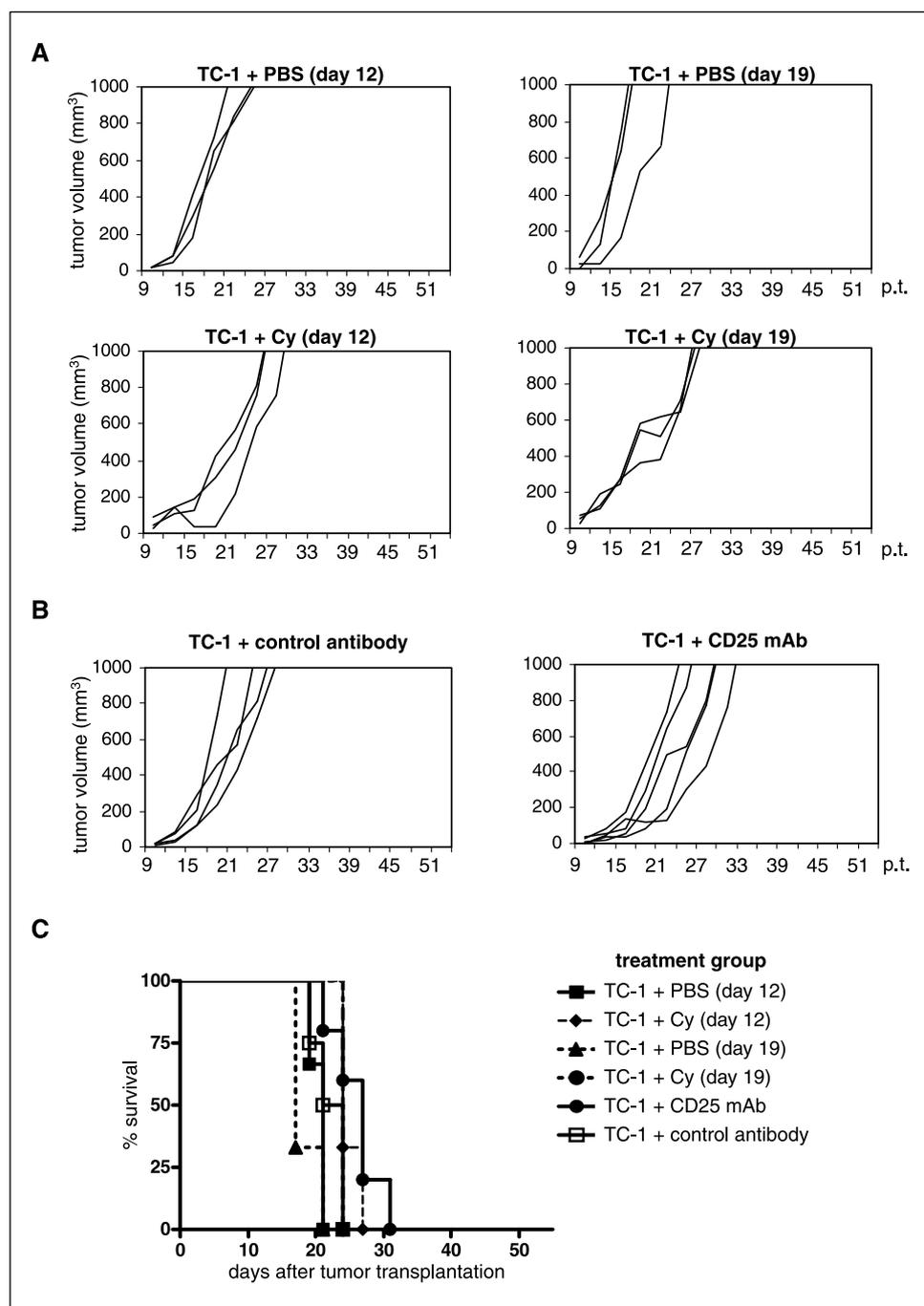
To evaluate murine tumor models for vaccination studies, we quantified TILs in TC-1 tumors. TC-1 cells are immortalized,



**Figure 2.** Presence of Tregs and CD8<sup>+</sup> T cells in TC-1 tumors and effect of cyclophosphamide and anti-CD25 mAbs on splenocytes. *A*, immunofluorescence analysis of Tregs in TC-1 tumors: cryosections of TC-1 tumors were analyzed by immunofluorescence staining. Characteristic section. Magnification,  $\times 40$ . FoxP3 staining appears red and is mostly localized to the cell center. CD25 staining appears green and is mostly localized to the cell periphery. *B*, flow cytometry analysis of Tregs in spleen and tumor. Three mice were s.c. injected with  $5 \times 10^4$  TC-1 cells. After 14 d, tumors were harvested and TILs were extracted and analyzed by flow cytometry using anti-FoxP3-PE, anti-CD4-PE, anti-CD25-FITC, and anti-CD8-PE antibodies. Characteristic analyses of TIL. *C*, effect of cyclophosphamide (Cy) injection on splenic Tregs. Mice were i.p. injected with 50, 100, or 200 mg/kg cyclophosphamide or PBS as a control (four animals per group). Four days later, spleens were harvested and weighed, total splenocytes were counted, and percentage of different subpopulations was determined by flow cytometry using anti-CD4-PE, anti-CD25-FITC, anti-CD8-PE, anti-CD19-PE, and anti-CD11b-PE antibodies. Total numbers of splenocyte subpopulations were calculated. *D*, effect of anti-CD25 antibody injection on splenic Tregs. Mice were i.p. injected with 500  $\mu$ g anti-CD25 (clone PC61.5) or control antibody (four animals per group). After 4 d, spleens were harvested, total splenocytes were counted, and percentage of different subpopulations was determined by flow cytometry using anti-CD4-PE and anti-CD25-FITC (clone 7D4).

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**Figure 3.** Effect of anti-CD25 mAbs and cyclophosphamide on TC-1 tumor growth. **A**, mice were s.c. injected with  $5 \times 10^4$  TC-1 cells in the right flank. On day 12 post-transplantation (*p.t.*; *left*) or day 19 (*right*) after tumor transplantation, mice received a single i.p. injection of 100 mg/kg cyclophosphamide or PBS as a control and tumor growth was followed. *Lines*, individual animals (three mice per group). This experiment was repeated once with a similar outcome. **B**, mice were s.c. injected with  $5 \times 10^4$  TC-1 cells. Five hundred micrograms anti-CD25 antibody or control antibody was injected i.p. on days -1, +2, and +4 after tumor injection. This experiment was repeated once with a similar outcome. **C**, survival curve of the mice described in (A) and (B).



murine epithelial cells that express the HPV type 16 proteins E6 and E7 as TAAs. An effective immune response against TC-1 cells correlates in most tumor vaccination studies with the induction of cytolytic CD8<sup>+</sup> T cells (CTLs) specific for E7 epitopes. Immunohistochemistry studies of TC-1 tumor sections revealed high numbers of intratumoral CD25<sup>+</sup>FoxP3<sup>+</sup> cells, indicating the presence of Tregs (Fig. 2A). In a quantitative approach, we extracted total TILs from tumors and analyzed these cells using flow cytometry (Fig. 2B). Surprisingly, we found that ~50% of intratumoral CD4<sup>+</sup> cells were CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>. For comparison, the relative Treg fraction in spleen (Fig. 2B) and peripheral blood cells (data not shown) was only ~10% of CD4<sup>+</sup> cells. This indicates that TC-1 tumors

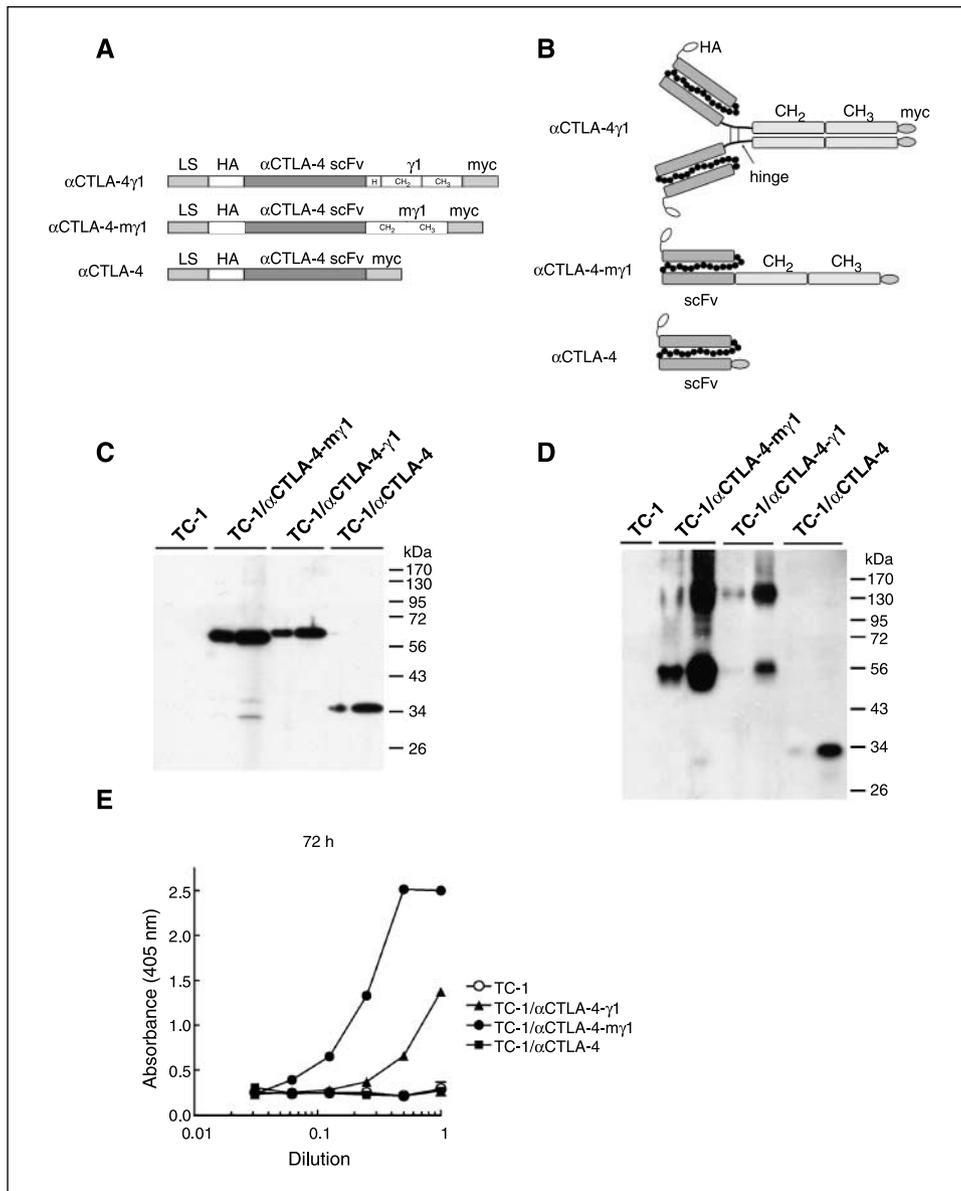
selectively attract/induce Tregs, which could be a possible mechanism to escape an antitumor immune response. Total CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T-cell numbers were  $4.5 \times 10^4 \pm 8 \times 10^3$ /g tumor. CD8 staining revealed the presence of CTLs in TC-1 tumors (total CD8<sup>+</sup> T cells,  $2.6 \times 10^4 \pm 7.9 \times 10^3$ /g tumor). Thus, the ratio of Treg/CD8 in TC-1-TIL is ~1.7, which indicates a quantitative increase of Tregs and is in line with our findings of TIL in human breast and cervical cancer.

**Effect of systemic Treg depletion on TC-1 tumor growth.** Given the potential role of Tregs in suppressing an immune response against TC-1 cells, we investigated whether systemic Treg depletion would affect the growth of TC-1 tumors. To deplete

Tregs, we used two previously reported methods, i.p. low-dose cyclophosphamide or i.p. anti-CD25 antibody injection. Cyclophosphamide was applied at a dose that selectively depleted Tregs. Cyclophosphamide was administered at two different time points after tumor transplantation to investigate whether Treg depletion would have an effect in the early (day 12) or late (day 19) growth phase of TC-1 tumors. Because earlier studies have suggested various cyclophosphamide doses (range, 30–200 mg/kg; refs. 18, 19) for Treg depletion in mice, we did a dose-response study. For this purpose 50, 100, or 200 mg/kg cyclophosphamide (or PBS as a control) was injected i.p. into naive mice. Splenocyte fractions were evaluated 4 days later by flow cytometry (previous publications have shown that the maximum Treg decrease occurs on day 4 after cyclophosphamide application; ref. 18). We found that 100 mg/kg cyclophosphamide decreased Treg levels significantly (~40%), whereas CD8<sup>+</sup> T cells were not significantly affected (Fig. 2C). A dose of 200 mg/kg cyclophosphamide was generally immunosuppressive, decreasing the number of all splenocyte fractions

investigated (CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD8<sup>+</sup>, CD11b<sup>+</sup>, and CD19<sup>+</sup>). Surprisingly, CD19<sup>+</sup> cells were most sensitive to cyclophosphamide, showing decreased levels at doses as low as 50 mg/kg cyclophosphamide. Taken together, low-dose cyclophosphamide treatment (100 mg/kg) selectively reduces Treg levels (when compared with CD8<sup>+</sup> cells) but also severely depletes CD19<sup>+</sup> lymphocyte levels in mice. Low-dose cyclophosphamide treatment (100 mg/kg) therefore could inhibit humoral adaptive immune responses (besides reducing numbers of Tregs), which has to be considered when using this agent.

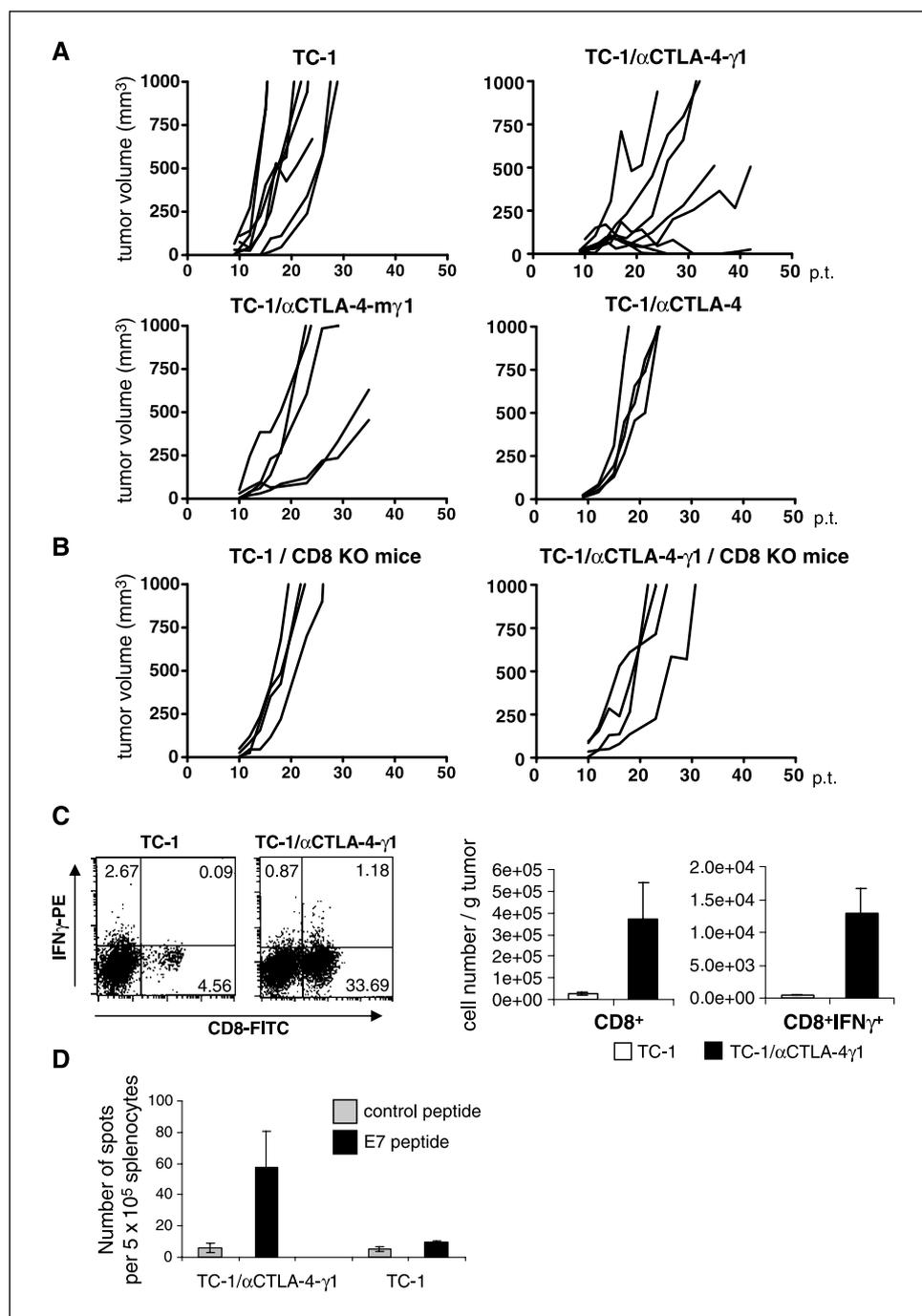
Because cyclophosphamide also induced multiple other immune changes, in addition to Treg depletion, we used anti-CD25 antibodies as a second approach for Treg depletion. A monoclonal anti-CD25 antibody (clone PC61.5; 500 µg) was injected i.p. on days -1, +2, and +4 after tumor cell injection. The PC61.5 antibody clone has been shown previously to deplete/inhibit Treg cells, when used at this dose (16). However, correct timing of anti-CD25 antibody application is important because CD25 is also expressed on



**Figure 4.** Generation and *in vitro* analysis of TC-1 cells. **A**, the  $\alpha$ CTLA-4- $\gamma$ 1 construct codes for an immunoglobulin V<sub>K</sub> signal peptide, an HA epitope, the anti-CTLA-4 scFv, the hinge, CH<sub>2</sub> and CH<sub>3</sub> domains of human IgG1, and a myc epitope.  $\alpha$ CTLA-4- $m\gamma$ 1 is similar but lacks the hinge region of human IgG1.  $\alpha$ CTLA-4 codes for a monomeric scFv. **B**, predicted structures of the scFv.  $\alpha$ CTLA-4- $\gamma$ 1 is predicted to form disulfide-linked dimers. **C** and **D**, TC-1 cells and derivatives were boiled in reducing (**C**) or nonreducing (**D**) SDS sample buffer, and lysate corresponding to  $0.2 \times 10^5$  (**left**) or  $1.3 \times 10^5$  (**right**) cells was resolved by SDS-PAGE and immunoblotted for the presence of the HA epitope (see Supplementary Data for details). **E**, established TC-1 cells expressing  $\alpha$ CTLA-4 scFv,  $\alpha$ CTLA-4- $m\gamma$ 1, or  $\alpha$ CTLA-4- $\gamma$ 1 were cultured for 72 h. Serial dilutions of the culture medium were then assayed for binding to recombinant CTLA-4 protein coated in microtiter plates by ELISA. Note that the culture medium from TC-1/ $\alpha$ CTLA-4- $m\gamma$ 1 cells showed a higher CTLA-4 binding capacity, which was most likely due to higher transgene expression levels in TC-1/ $\alpha$ CTLA-4- $m\gamma$ 1 cells (see **C** and **D**). In contrast, culture medium from TC-1/ $\alpha$ CTLA-4 cells did not show significant CTLA-4 binding, although binding was detected in culture medium collected after 48 h (data not shown). We speculate that this is due to poor stability of the anti-CTLA-4 scFv protein.

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**Figure 5.** Effect of anti-CTLA-4 antibody expression on antitumor immune responses. **A**, effect of local anti-CTLA-4 antibody expression. Immunocompetent mice were s.c. injected with  $5 \times 10^4$  TC-1 cells or established TC-1 cells expressing  $\alpha$ CTLA-4 scFv,  $\alpha$ CTLA-4-m $\gamma$ 1, or  $\alpha$ CTLA-4- $\gamma$ 1 and tumor growth was followed. Treatment groups contained 4 mice (TC-1/ $\alpha$ CTLA), 6 mice (TC-1/ $\alpha$ CTLA-4 m $\gamma$ 1), or 8 mice (TC-1; TC-1/ $\alpha$ CTLA-4- $\gamma$ 1). This experiment was repeated twice with a similar outcome. **B**, role of immune cells. CD8 KO mice were s.c. injected with  $5 \times 10^4$  TC-1 or TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells (four animals per group). **C**, intracellular cytokine staining for CD8 and IFN $\gamma$ . Immunocompetent mice were s.c. injected with  $5 \times 10^4$  TC-1 or TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells (three animals per group). Tumors were harvested after 21 d and TILs were extracted and immediately analyzed by flow cytometry using anti-CD8-FITC and anti-IFN $\gamma$ -PE antibodies. *Left*, characteristic analyses of IFN $\gamma$ -producing CD8 $^+$  cells; *right*, total numbers of TIL subpopulations per gram tumor. **D**, splenocytes from mice, which showed TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumor regression, were harvested 21 d after tumor transplantation (control group: TC-1 tumor-bearing mice, 21 d after tumor transplantation) and subsequently analyzed for frequencies of IFN $\gamma$ -producing E7-specific T cells via ELISPOT as described previously (31). Frequencies of IFN $\gamma$ -producing T cells specific to the HPV16 E7<sub>49-57</sub> H-2D<sup>p</sup>-restricted peptide (RAHYNIVTF) or an unrelated control peptide (three animals per group).



activated T effector cells. Therefore, anti-CD25 antibody injection was scheduled at the beginning of the vaccination treatment, to prevent possible depletion of the activated T effector subpopulation. To investigate the effect of anti-CD25 antibody on Tregs, splenocytes of naive mice were extracted 4 days after application of 500  $\mu$ g antibody and stained for CD4 $^+$ CD25 $^+$  cells (using an antibody targeted toward a different CD25 epitope) and other lymphocyte subpopulations (CD8 $^+$ , CD11b $^+$ , and CD19 $^+$ ). Anti-CD25 antibody decreased the numbers of CD4 $^+$ CD25 $^+$  lymphocytes by  $\sim$ 60% (Fig. 2D), whereas CD8 $^+$ , CD11b $^+$ , and CD19 $^+$  cell levels were not affected (data not shown), indicating that anti-CD25 antibody treatment is a specific approach for Treg depletion.

As shown in Fig. 2C and D, both low-dose cyclophosphamide and anti-CD25 antibody treatment exerted only partial depletion effects on Tregs ( $\sim$ 40% and  $\sim$ 60%, respectively). However, both agents are also known to inhibit Treg function (without depleting these cells; refs. 9, 18). Therefore, the resulting Treg inhibition might be considerably greater than indicated by the cell deletion data. When given as a single agent, both low-dose cyclophosphamide and anti-CD25 antibody treatment had slight but not significant effects on TC-1 tumor growth (Fig. 3). We therefore conclude that Treg depletion/inhibition alone (using the described approaches) is not sufficient to induce efficient antitumor immune responses against TC-1 tumor cells.

**Systemic CTLA-4 blockade induces organ-specific autoimmune responses but no anti-TC-1 tumor immune responses.** It has been shown in various cancer models that systemic anti-CTLA-4 antibody administration results in the induction of therapeutic antitumor immune responses. To evaluate the effect of CTLA-4 blocking antibodies on TC-1 tumors, we i.p. administered 150  $\mu$ g anti-CTLA-4 antibody or control antibody at days -4, -2, 0, 2, 4, 7, 10, 14, 17, and 25 (1.5 mg total) after TC-1 tumor transplantation (five animals per group). Although anti-CTLA-4 antibodies were functional (Supplementary Fig. S1), no significant antitumor effect of systemic CTLA-4 blockade on tumor growth was observed (data not shown). However, all animals that received systemic CTLA-4 blockade developed spontaneous organ-specific autoimmune reactions reflected by mononuclear cell infiltration in the liver within 30 days. Mice injected with the same dose of control antibody also showed hepatitis, but to a lesser degree (Supplementary Fig. S2). All other organs investigated (heart, lung, brain, liver, kidney, stomach, mesenterium, muscle, and skin) showed normal histology in mice, which were systemically treated with anti-CTLA-4 or control antibody.

**Effect of anti-CTLA-4 antibody expression from TC-1 cells on antitumor immune response and tumor growth.** Because TC-1 tumors attract Tregs and CTLs, we hypothesized that production of CTLA-4 antibodies from tumor cells would locally activate tumor-reactive CTLs, either directly, by blocking CTLA-4 on CTLs, or indirectly, by inhibiting Treg function. We therefore generated TC-1 cells that stably express either (a) an anti-CTLA-4 scFv fused to a human Fc fragment (TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells), (b) an anti-CTLA-4 scFv fused to a human Fc fragment lacking the hinge region (TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1), or (c) an  $\alpha$ CTLA-4 scFv alone (TC-1/ $\alpha$ CTLA-4; Fig. 4A and B). Expression of the respective transgenes was confirmed in TC-1 cell lysates by Western blot (anti-HA tag; Fig. 4C and D). As predicted,  $\alpha$ CTLA-4- $\gamma$ 1 predominantly forms dimers, whereas  $\alpha$ CTLA-4-m $\gamma$ 1 (which lacks the hinge region) is mostly present as a monomer. To evaluate the ability of the different antibody constructs to bind CTLA-4, the same number of cells was seeded and culture medium was collected after 72 h. As determined by ELISA, culture medium from TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 and TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1 cells showed significant CTLA-4 binding (compared with native TC-1 cells), which shows that the respective antibodies are secreted (Fig. 4E, similar results after 96 h; data not shown).

To evaluate the effect of local anti-CTLA-4 antibody expression on immune responses *in vivo*,  $5 \times 10^4$  TC-1, TC-1/ $\alpha$ CTLA-4- $\gamma$ 1, TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1, or TC-1/ $\alpha$ CTLA-4 cells were injected s.c. into syngeneic C57Bl/6 mice. As expected, expression of the  $\alpha$ CTLA-4 scFv fragment (TC-1/ $\alpha$ CTLA-4) had no apparent effect on tumor growth (no significant delay in tumor growth compared with TC-1 cells; Fig. 5A). In contrast, expression of the functional CTLA-4 antibody ( $\alpha$ CTLA-4- $\gamma$ 1) had a pronounced effect on TC-1 tumor growth, causing transient tumor regression in 3 of 8 mice and permanent tumor regression in 2 of 8 mice (Fig. 5A), accompanied by a significant increase in average survival time (median survival of the treatment groups: TC-1, 23.5 days; TC-1/ $\alpha$ CTLA-4- $\gamma$ 1, 42 days;  $P < 0.0005$ ). TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1 cells showed slightly delayed outgrowth (median survival, 29 days), which was not significant when compared with the TC-1 control group ( $P < 0.07$ ). This is surprising because TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1 cells produced more recombinant antibody protein and the CTLA-4 binding capacity of the culture medium of these cells was higher when compared with TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells. In contrast to local anti-CTLA-4

antibody expression, i.e. injection of anti-CTLA-4 antibody (200  $\mu$ g) had no antitumor effect (when compared with control antibody-treated group; five animals per group; data not shown). This indicates that continuous CTLA-4 blockade (via anti-CTLA-4 antibody expression inside the tumor) is more effective compared with transient CTLA-4 blockade (via one time injection of the anti-CTLA-4 antibody). Because local tumor production of the CTLA-4- $\gamma$ 1 antibody apparently triggered the strongest antitumor effect, the following studies focused on TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells. To dissect whether immunologic or direct cytotoxic effects are responsible for the effect of anti-CTLA-4 antibody production on TC-1 tumor growth,  $5 \times 10^4$  TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells were injected s.c. into syngeneic SCID (data not shown) or CD8 KO mice (Fig. 5B). No significant delay in TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumor growth was observed in these animals when compared with normal TC-1 cells. The absence of a delay in the growth of TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells in SCID and CD8 KO mice indicates that immune cells (particularly CD8<sup>+</sup> CTLs) are responsible for the observed tumor regression in immunocompetent mice. To further study the nature of these immune cells, TC-1 and TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors were established in immunocompetent C57Bl/6 mice, and TILs were extracted after 21 days and immediately processed for flow analysis (Fig. 5C). The average number of total CD8<sup>+</sup> cells in TC-1 tumors was  $2.6 \times 10^4 \pm 7.9 \times 10^3$ /g tumor. IFN $\gamma$ -producing CD8<sup>+</sup> cells were barely detectable in TC-1 tumors ( $512 \pm 34$  CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells/g tumor). In contrast, TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors showed a ~14-fold increase of CD8<sup>+</sup> cells compared with TC-1 tumors (mean,  $3.7 \times 10^5 \pm 1.6 \times 10^5$  CD8<sup>+</sup> cells/g tumor). Furthermore, the number of tumor-infiltrating CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells increased ~25-fold in anti-CTLA-4 antibody-producing TC-1 tumors ( $1.3 \times 10^4 \pm 3.8 \times 10^3$  CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells/g tumor). Because the number of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells did not significantly change (data not shown), the Treg/CD8 ratio in TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors was ~0.25, whereas TC-1 tumors showed an average Treg/CD8 ratio of ~1.7. Thus, local expression of the functional CTLA-4 antibody changes the intratumoral Treg/CD8 ratio toward an increase of CD8<sup>+</sup> T cells and greatly increases the intratumoral number of activated CD8<sup>+</sup> cells. TC-1/ $\alpha$ CTLA-4-m $\gamma$ 1 tumors also showed significantly increased numbers of activated CD8<sup>+</sup> cells although significantly less than TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors ( $6.7 \times 10^4 \pm 1.4 \times 10^4$  CD8<sup>+</sup> cells/g tumor and  $1.7 \times 10^3 \pm 245$  CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells/g tumor), which correlates with the less pronounced effect of  $\alpha$ CTLA-4-m $\gamma$ 1 expression on TC-1 tumor growth in immunocompetent mice (Fig. 5A). Animals with complete TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumor regression showed significantly higher frequencies of splenic HPV-16 E7-specific, IFN $\gamma$ -producing T cells compared with TC-1 tumor-bearing mice as determined by enzyme-linked immunospot (ELISPOT) analysis 21 days after tumor transplantation (Fig. 5D). These data indicate that tumor site-located CTLA-4 blockade can enhance local and systemic antitumor immune responses.

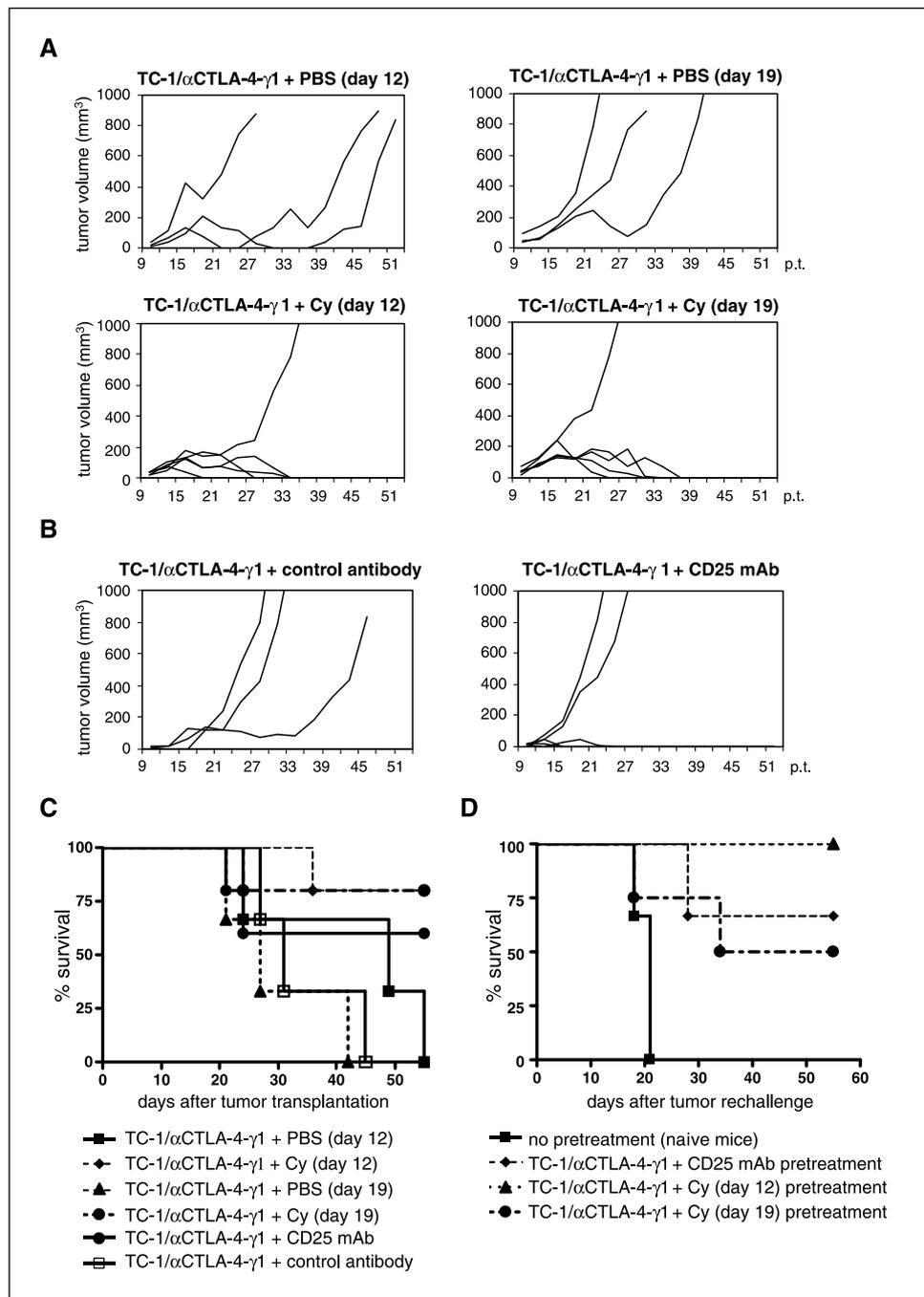
**TC-1 tumor regression and antitumor immunity following combined Treg depletion and anti-CTLA-4 antibody treatment.** The observed activating effect of anti-CTLA-4 antibody expression on tumor-infiltrating CD8<sup>+</sup> cells could be due to inhibition of Tregs or direct activation of CTLs (see Introduction). To dissect the mechanism of tumor regression induced by local CTLA-4 antibody expression, we investigated whether Treg depletion would have an additional effect on TC-1 tumor regression. Importantly, when systemic Treg depletion (using either low-dose cyclophosphamide or CD25 mAb) was combined

with local  $\alpha$ CTLA-4 expression (TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells), efficient tumor regression was observed in 3 of 5 mice (CD25 mAb), 4 of 5 mice (low-dose cyclophosphamide, day 12), and 4 of 5 mice (low-dose cyclophosphamide, day 19;  $P < 0.05$  compared with PBS or control antibody treatment groups, respectively, log-rank test; Fig. 6A-C). Long-term tumor eradication was seen (no regrowth within 55 days of observation). In contrast, local anti-CTLA-4 expression as a single treatment resulted in only transient tumor regression in the majority of animals (see Fig. 5A) and application of Treg-depleting agents alone (anti-CD25 antibody or low-dose cyclophosphamide) had no significant effect on tumor growth (see Fig. 2C and D). Thus, the combination of both local CTLA-4

blockade and systemic Treg depletion had a synergistic effect on TC-1 tumor regression.

Next, we tested whether mice that showed long-term tumor regression were also protected against a tumor rechallenge. Animals, with no tumor regrowth within 55 days of combination treatment (see Fig. 6C), were s.c. injected with  $2.5 \times 10^4$  normal TC-1 cells on both flanks (Fig. 6D). Naive mice (of the same age), which received the same tumor cell dose, showed tumor outgrowth on both flanks (average survival time, 21 days). In contrast, the majority of mice [2 of 3, TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 + anti-CD25 mAb pretreatment group; 4 of 4 TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 + cyclophosphamide (day 12) pretreatment group; and 2 of 4 TC-1/

**Figure 6.** Effect of local anti-CTLA-4 antibody expression and systemic Treg repression by low-dose cyclophosphamide or anti-CD25 mAb treatment on TC-1 tumor growth. **A**, tumor growth after combined anti-CTLA-4/cyclophosphamide treatment. Mice were s.c. injected with  $5 \times 10^4$  TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells in the right flank. On day 12 and 19 post-transplantation, mice received a single i.p. application of 100 mg/kg cyclophosphamide or PBS as a control. Three animals [PBS (day 12), PBS (day 19)] and five animals per group [cyclophosphamide (day 12), cyclophosphamide (day 19)] were used. This experiment was repeated once with a similar outcome. **B**, tumor growth after combined anti-CTLA-4/anti-CD25 treatment. Mice were s.c. injected with  $5 \times 10^4$  TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells. Five hundred micrograms anti-CD25 antibody or control antibody was injected i.p. on days -1, +2, and +4 after tumor injection. Three animals (control antibody) and five animals (CD25 mAb) per treatment group were used. This experiment was repeated once with a similar outcome. **C**, survival curve of the mice described in (A) and (B). Mice, which showed tumor regression (and no relapse in tumor growth) on combination treatment in (A) and (B), were rechallenged with TC-1 cells on day 55 after injection of primary TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells. TC-1 cells ( $2.5 \times 10^4$ ) were s.c. injected in both flanks of the mice and tumor growth was followed for 55 d. Mice were sacrificed when tumors reached a size of 1,000 mm<sup>3</sup>. Four animals [TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 + cyclophosphamide (day 12) pretreatment group and TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 + cyclophosphamide (day 19) pretreatment group] and three animals (naive mice, TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 + CD25 mAb pretreatment group) per treatment group were used. This experiment was repeated once with a similar outcome.



$\alpha$ CTLA-4- $\gamma$ 1 + cyclophosphamide (day 19) pretreatment group], which received previously the combination treatment, showed no tumor outgrowth on TC-1 rechallenge within an observation period of 2 months. This indicates that combined treatment using local CTLA-4 blockade together with either low-dose cyclophosphamide or anti-CD25 antibody efficiently induced protective immunity against TC-1 cells in these animals.

**Absence of autoimmune reactions.** Autoimmunity after systemic application of anti-CTLA-4 antibodies has been reported with characteristic inflammation of lung, liver, intestine, and stomach accompanied by glomerular deposition of antibodies in the kidneys (32). To assess autoimmune reactions in our approach, organs and skin of TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumor-bearing mice were collected, paraffin embedded, and sectioned for H&E staining (Supplementary Figs. S2 and S3). In contrast to animals that received systemic anti-CTLA-4 antibody injection, no evidence of inflammatory processes (e.g., mononuclear infiltrations) was detectable in mice that received TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells. Furthermore, immunohistochemistry staining for IgG complexes on kidney sections did not reveal abnormalities (data not shown). In addition, no evidence of autoimmune reactions was detectable in mice that received TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 cells in combination with low-dose cyclophosphamide or anti-CD25 antibodies.

## Discussion

Increasing evidence indicates that T-cell-mediated mechanisms of immune tolerance, especially CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs, are present in human tumors and locally suppress antitumor T-cell responses, thereby allowing the tumor to escape immunosurveillance (2–6).

In breast cancer patients with advanced disease, the tumor microenvironment, draining lymph nodes, and pleural effusions contain high numbers of Tregs (5, 33, 34), which potently suppressed the proliferation and IFN $\gamma$  secretion of autologous activated CD8<sup>+</sup> cells *in vitro* (5). Here, we corroborate these previous findings, showing high numbers of Tregs in the tumor stroma of breast cancer patients with stage III and IV disease (Fig. 1A; Supplementary Table S1). Because CD8<sup>+</sup> T cells were colocalized with Tregs in the tumor stroma, both cell-cell contact and cytokine-dependent mechanisms of Treg-mediated CD8<sup>+</sup> T-cell inhibition could contribute to a potential intratumoral immunosuppression.

For human cervical cancer, we show for the first time that intratumoral Tregs are abundantly present. The Treg cell number was significantly higher in cervical cancer compared with normal tissue. Tregs were also frequently detectable in precancerous lesions (CIN1, CIN2, and CIS), indicating a possible role of Tregs not only in the progression but also in the development of cervical cancer. Because the vast majority of cervical cancers are associated with HPV infections, Tregs may play a role in creating a local immunoprivileged site to facilitate the survival of HPV-infected cells. A role of Tregs in other chronic virus infections, such as hepatitis C virus and human immunodeficiency virus, has been suggested recently (35).

In patients, systemic anti-CTLA-4 antibody treatment has been shown not only to activate tumor-specific T-cell responses against melanomas (which typically contain Tregs; ref. 4) but also to induce autoimmunity (36, 37). We hypothesized that local anti-CTLA-4 antibody expression would be sufficient to overcome local immune tolerance mechanisms in tumors while reducing the risk of autoimmune responses. We studied vaccination strategies

using TC-1 mouse tumors, which selectively attract Tregs and CD8<sup>+</sup> T cells at a ratio similar to human cancer. To analyze the effect of local anti-CTLA-4 antibody production on antitumor immune responses, we established TC-1 cells, which stably express a functional anti-CTLA-4 antibody (TC-1/ $\alpha$ CTLA-4- $\gamma$ 1). When injected into immunocompetent mice, TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors regressed in the majority of mice, which was accompanied by increased numbers of tumor-infiltrating IFN $\gamma$ -producing CD8<sup>+</sup> T cells, a concomitant decrease of the intratumoral Treg/CD8 ratio and significantly prolonged survival time compared with control tumors. Together with the fact that TC-1/ $\alpha$ CTLA-4- $\gamma$ 1 tumors grew normally in SCID and CD8 KO mice (Fig. 5B), this indicates that tumor regression by local anti-CTLA-4 antibody is dependent on the immune system, particularly CTLs. Importantly, no signs of autoimmune reactions were seen in our TC1-1/C57Bl/6 model with local production of anti-CTLA-4 antibody. In contrast, systemic anti-CTLA-4 antibody application has been shown to exacerbate/induce autoimmunity in mice (14, 38) and patients (36, 37). We have confirmed this observation in our studies in mice. In contrast to systemic anti-CTLA-4 antibody application, tumor-localized expression of anti-CTLA-4 was both more effective and safe. This indicates that continuous intratumoral production of anti-CTLA-4 is superior to systemic application of anti-CTLA-4 antibodies to overcome local mechanisms of immune tolerance and activate tumor-reactive lymphocyte fractions inside the tumor and tumor-draining lymph nodes. We also speculate that the restriction of T-cell activation to the site of the tumor and tumor-draining lymph nodes explains the absence of autoimmune reactions compared with systemic anti-CTLA-4 application.

It is currently disputed by which mechanism(s) anti-CTLA-4 antibody treatment induces autoreactive and tumor-reactive T cells. Because CTLA-4 is expressed on two different lymphocyte compartments (Tregs and activated T effector cells), two major mechanisms of immunoactivation by CTLA-4-blocking antibodies have been proposed: inhibition of Tregs or direct activation of T effector cells. Several groups have suggested a predominant effect of CTLA-4 antibodies toward T effector cells, whereas Treg function is not affected (28, 38, 39). In line with these reports, our study indicates that CTLA-4 antibody treatment and Treg depletion alter different regulatory pathways *in vivo*. This is further supported by our finding that the combination of tumor-localized anti-CTLA-4 expression with methods of systemic Treg depletion had a synergistic effect. Synergy between CTLA-4 blockade and Treg depletion on immune responses has also been observed in two other studies. Suttmuller et al. (38) reported that systemic application of both anti-CD25 and anti-CTLA-4 antibodies efficiently induced CTL-mediated regression of B16 melanomas. However, in this study, all mice that received the combined antibody treatment also developed autoimmunity. Takahashi et al. (14) also reported development of autoimmune disease (in particular autoimmune gastritis) in mice that received combined systemic anti-CD25 and anti-CTLA-4 antibody application. Importantly, we did not find autoimmune reactions when anti-CTLA-4 antibody is expressed in tumors and anti-CD25 antibodies are given *i.p.*

Clearly, studies in other mouse tumor models and, eventually, in cancer patients are required to validate our findings in the TC-1 tumor model. TC-1 cells express HPV E6 and E7 proteins, which could skew the immune response toward these non-self-antigens and mask potential autoimmune responses. Therefore, we are

currently doing studies with recombinant HER-2 (rHER-2)/*neu* transgenic mice and syngeneic rHER-2/*neu*-positive tumors. Furthermore, given the side effects of cyclophosphamide on multiple components of the immune system, we will use anti-CD25 or anti-GITR antibodies for Treg depletion/inhibition in our further studies with tumor-localized anti-CTLA-4 expression.

This is to our knowledge the first study showing that local intratumoral CTLA-4 blockade (as a single treatment or in combination with Treg depletion) exerts effective antitumor immune responses and is not associated with adverse systemic effects (especially autoimmunity) in mice. We believe that tumor site-located CTLA-4 blockade, especially when combined with Treg depletion, represents a promising new approach for

immunotherapy of cancer, specifically of naturally immunogenic tumors that induce/attract TAA-specific CTLs and Tregs (e.g., breast cancer, ovarian cancer, cervical cancer, and melanoma).

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