The Requirement of Multimodal Therapy (Vaccine, Local Tumor Radiation, and Reduction of Suppressor Cells) to Eliminate Established Tumors

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Abstract

Purpose: Numerous immune-based strategies are currently being evaluated for cancer therapy in preclinical models and clinical trials. Whereas many strategies look promising in preclinical models, they are often evaluated before or shortly following tumor implantation. The elimination of well-established tumors often proves elusive. Here we show that a multimodal immune-based therapy can be successfully employed to eliminate established tumors.

Experimental Design: This therapy consists of vaccines directed against a self-tumor-associated antigen, the use of external beam radiation of tumors to up-regulate Fas on tumor cells, and the use of a monoclonal antibody (mAb) to reduce levels of CD4+CD25+ suppressor cells.

Results: We show here for the first time that (a) antigen-specific immune responses induced by vaccines were optimally augmented when anti-CD25 mAb was given at the same time as vaccination; (b) anti-CD25 mAb administration in combination with vaccines equally augmented T-cell immune responses specific for a self-antigen as well as those specific for a non–self antigen; (c) whereas the combined use of vaccines and anti-CD25 mAb enhanced antigen-specific immune responses, it was not sufficient to eliminate established tumors; (d) the addition of external beam radiation of tumors to the vaccine/anti-CD25 mAb regimen was required for the elimination of established tumors; and (e) T cells from mice receiving the combination therapy showed significantly higher T-cell responses specific not only for the antigen in the vaccine but also for additional tumor-derived antigens (p53 and gp70).

Conclusions: These studies reported here support the rationale for clinical trials employing multimodal immune-based therapies.

Numerous cancer vaccines are being investigated targeting various tumor-associated self-antigens. CD4+CD25+ immunosuppressive/immunoregulatory T (Treg) cells have been implicated in the maintenance of immunologic tolerance to self-antigens. Clinical studies have shown an increase in levels of CD4+CD25+ cells in cancer patients (1–9), suggesting that the increment of these cells is correlative to the stage of cancer progression. Therefore, it has been hypothesized that Treg cell depletion may improve antitumor efficacy with the use of cancer vaccines. It has been shown in murine tumor models that either (a) depletion of CD4+CD25+ cells by anti-CD25 monoclonal antibody (mAb) administration (10–12), or (b) adoptive cell transfer after CD4+CD25+ cell depletion (13, 14), could result in significant antitumor activity. In addition, depletion of Treg cells via anti-CD25 mAb administration has been combined with whole tumor cell vaccine therapy (15) and peptide vaccine therapy (16). In these studies, however, anti-CD25 mAb was given several days before or only 1 day after tumor implantation. No reports exist as to whether anti-CD25 mAb administration could increase antitumor efficacy induced by therapy of more established tumors.

We previously developed recombinant poxviral vectors that contain the transgenes for a carcinoembryonic antigen (CEA), a triad of T-cell costimulatory molecules (B7-1, intracellular adhesion molecule [ICAM-1], LFA-3; designated TRICOM), and the combination of the four transgenes (CEA/TRICOM). Two types of poxvirus vectors were developed: replication-competent recombinant vaccinia (rV) and replication-defective recombinant fowlpox (rF; refs. 17–19). In the study reported here, we first examine whether anti-CD25 mAb administration can enhance antigen-specific T-cell responses and antitumor immunity induced by CEA/TRICOM vaccines. In these studies, however, anti-CD25 mAb was given several days before or only 1 day after tumor implantation. No reports exist as to whether anti-CD25 mAb administration could increase antitumor efficacy induced by therapy of more established tumors.

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Materials and Methods

Animals and tumor cells. Female C57Bl/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Female C57Bl/6 mice transgenic for human CEA were obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Germany). The generation and characterization of the CEA-transgenic mouse has been previously described (20, 21). Mice were housed and maintained under pathogen-free conditions in microisolator cages until used for experiments at 6 to 8 weeks of age.

The following murine tumor cell lines (H-2b) were used: lymphoma EL-4 cells, parental colon adenocarcinoma MC38 cells, MC38 expressing human CEA (designated MC38-CEA, ref. 22), and melanoma B16 cells (CEA: gp70). These cells except EL-4 cells were trypsinized, and all cells were washed in PBS before use.

Recombinant poxviruses. The recombinant vaccinia virus designated rV-CEA/TRICOM contains the murine B7-1, ICAM-1, and LFA-3 genes in combination with the human gene CEA as described elsewhere (23). The recombinant fowlpox virus encoding the same genes was designated rF-CEA/TRICOM (23). The recombinant vaccinia virus designated rV-LacZ/TRICOM was constructed in a similar manner and designated rF-LacZ/TRICOM. The recombinant fowlpox virus containing murine GM-CSF gene (designated rF-GM-CSF) was also used for vaccination (24). Therion Biologics Corp. (Cambridge, MA) kindly provided all orthopox viruses.

Antibodies and flow cytometric analysis. Anti-murine CD25 mAb (PC61) and the isotype rat IgG1 (R187) were purified from culture supernatants of each hybridoma cell line (American Type Culture Collection, Manassas, VA) using a protein G column for in vitro administration.

To analyze the percentage of CD4+CD25+ cells in mice, inguinal lymph nodes, spleens, and peripheral blood cells were prepared into single cell suspensions and RBC were removed for flow cytometric analysis. The following antibodies were purchased from BD PharMingen (San Diego, CA) and used for analysis: PE-conjugated anti-CD25 (3C7, rat IgG2b), CyChrome-conjugated anti-CD4 (rat IgG2a), FITC-conjugated anti-CD3e (hamster IgG1), PE-conjugated anti-CTLA-4 (hamster IgG1), anti-rat IgG2a to detect anti-GITR mAb-binding cells, each appropriate isotype control, and anti-CD16/CD32 mAb to block Fc receptors. Anti-GITR mAb was purchased from R&D Systems (Minneapolis, MN). To evaluate the generation of gp70-specific CTLs in mice, cells were stained with FITC-conjugated anti-CD3e, CyChrome-conjugated anti-CD8 mAb (rat IgG2a) and PE-conjugated p15E04-611/H-2Kb-tetramer (called gp70-tetramer) obtained from the NIH Tetramer Facility (25). Cell samples from blood and spleens of naive mice were tested as a control for this tetramer assay, and 2% to 3% of these cells were positive in the CD8+gp70-tetramer+ fraction after gating CD3e.

Immunofluorescence staining was done after Fc receptor-blocking with anti-CD16/CD32 mAb (30 minutes on ice). Cells were incubated with antibodies or gp70-tetramer for 30 minutes on ice and washed with 1% bovine serum albumin/PBS thrice. The immunofluorescence was compared with the appropriate isotype-matched controls and analyzed with Cellquest software using a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA).

Treatments with TRICOM vaccines and anti-CD25 mAb. In protocols for assays to determine the timing for anti-CD25 combination with rV-CEA/TRICOM, C57Bl/6 mice were vaccinated s.c. with rV-CEA/TRICOM [1 × 107 plaque-forming units (pfu)/mouse] admixed with rF-GM-CSF (107 pfu/mouse) on day 0. These mice were injected i.p. with anti-CD25 mAb (300 μg/mouse) on day −8, −4, −2, 0, 1, 2, 4, or day 8. In protocols for assays to examine immune responses to a self-antigen and a foreign antigen at the same time, CEA-transgenic mice were vaccinated s.c. with the mixture of rV-CEA/TRICOM (5 × 107 pfu/mouse) and rLVac/LacZ/TRICOM (5 × 107 pfu/mouse) admixed with rF-GM-CSF (1 × 107 pfu/mouse), immediately after i.p. injection with anti-CD25 mAb (300 μg/mouse). These mice were sacrificed 14 days after vaccination, and splenic T cells were used for in vitro assays.

Lymphocyte proliferation assay. To evaluate CD4+ T-cell responses specific for antigens, splenic T cells were tested for proliferation in response to protein or peptide antigens as previously described (26). Briefly, pooled splenic T cells (1.5 × 106 cells per well) were cultured in 96-well flat-bottomed plates with irradiated naive syngeneic splenocytes as antigen-presenting cells (5 × 103 cells per well) and stimulants: human CEA protein (1.56-50 μg/mL, Aspen Bio, Littleton, CO), β-gal protein (1.56-50 μg/mL, Prozyme, San Leandro, CA), or p53105-122 class II peptide (0.16-5 μg/mL, LCFLQ5GTAKSMICT, ref. 27). As a positive control, cells were stimulated with a T-cell mitogen Concanavalin A (2.5 μg/mL, Sigma-Aldrich, St. Louis, MO). T cells and antigen-presenting cells were cultured with medium only as a negative (background) control. Cells were cultured for 5 days. 3H-thymidine (1 μCi/well) was added to the wells for the last 18 to 24 hours and harvested using a Tomtec cell harvester (Wallac, Inc., Gaithersburg, MD). The incorporated radioactivity was measured using a liquid scintillation counter (Wallac 1205 Betaplate, Wallac).

The mean proliferation of negative control responses was subtracted from proliferation in response to antigens: CEA protein, β-gal protein, or p53 class II peptide. The data were averaged and graphed as cpm ± SD. To evaluate interleukin 10 (IL-10) release from CD4+ T cells in response to these antigens, the supernatant was collected 48 hours after culture, and IL-10 concentration was measured using mouse IL-10 immunoassay kit (Biosource International, Camarillo, CA). Nonspecific IL-10 production cultured in medium only was subtracted from that induced by antigens.

Cytotoxicity assay. To evaluate CD8+ T-cell responses specific for antigens, first, spleens were pooled and dispersed into single cell suspensions and stimulated with the H-2Dk-restricted peptide CEA265-273 (10 μg/mL, EAQNTTYL; refs. 21, 28), the H-2Kb-restricted peptide β-gal491-500 (DAPHYTV; ref. 29), the H-2Db-restricted peptide p53212-220 (2 μg/mL, KYMCNSSCM; ref. 30), or the H-2Kb-restricted peptide p15E04-611 (1 μg/mL, KSPWFTTL, called gp70 peptide; ref. 25). Six days later, bulk lymphocytes were separated by centrifugation through a Ficoll-Hypaque gradient.

Using these recovered lymphocytes, tumor-killing activity was tested as described previously (26). Briefly, the recovered lymphocytes, 51Cr-labeled target tumor cells (EL-4, 5 × 104 cells per well), and each peptide were incubated for 5 hours (96-well U-bottomed plates), and radioactivity in supernatants was measured using a γ-counter (Corba Autogamma, Packard Instruments, Downers Grove, IL). As control peptides, VSV-N52-59 (RGYVYQGL; ref. 31) was used for H-2Db-restricted peptides, or ovalbumin257-264 (SIINFEKL; ref. 32) was used for H-2Kb-restricted peptides. In some experiments, MC38-CEA+ cells, the parental MC38 cells or B16 cells were used as a target without peptide. The percentage of tumor lysis was calculated as follows: % tumor lysis = [(experimental cpm – spontaneous cpm) / (maximum cpm – spontaneous cpm)] × 100. Nonspecific 51Cr release in response to appropriate control
peptide was subtracted from that induced by each tumor antigen peptide. The data were averaged and graphed as %Δ ± SD.

**Cytokine production assay.** The recovered lymphocytes separated as described above were also tested for cytokine production from CD8+ T cells in response to antigen peptides. Lymphocytes (5 × 10^6 cells per well) were restimulated with fresh irradiated naive splenocytes (5 × 10^6 cells per well) and each peptide: 10 μg/mL of CEA peptide, 10 μg/mL of β-gal peptide, 2 μg/mL of p53 peptide, or 1 μg/mL of gp70 peptide. Twenty-four hours later, the supernatant fluid was collected and analyzed for murine IFN-γ using the Cytometric Bead Array kit (BD Pharmingen). Non-specific IFN-γ production in response to appropriate control peptide was subtracted from that induced by each tumor antigen peptide.

**Combination therapy with CEA/TRICOM vaccines, anti-CD25 mAb, and/or γ radiation.** MC38-CEA+ tumor cells were implanted s.c. into the right leg of CEA-transgenic mice. Eight days after tumor implantation, mice were treated s.c. with rV-CEA/TRICOM and i.p. with anti-CD25 mAb (300 μg/mouse). In a protocol combining radiation therapy, the tumor-implanted legs were irradiated at 8 Gy on day 14 according to the method described elsewhere (33). The dose used (8 Gy) was predetermined to have a minimal effect on the growth rate of tumors implanted. Mice were then boosted with rf-CEA/TRICOM on days 15 and 22. As a late-phase therapy, the triple combination therapy was started on day 13; mice were treated s.c. with rf-VEA/TRICOM and i.p. with anti-CD25 mAb on day 13, irradiated at 8 Gy on day 19, and boosted with rf-CEA/TRICOM on day 20. Each virus was injected at 1 × 10^7 pfu/mouse admixed with 1 × 10^8 pfu/mouse of rf-GM-CSE. The size of solid tumors was measured using calipers one to two times a week. The tumor volumes were calculated as follows: tumor volume (mm^3) = 0.5 × length × width^2. Mice were sacrificed when either size (length or width) of tumors exceeded 20 mm.

In an indicated experiment, CD4+ T cells and/or CD8+ T cells were depleted from the mice receiving the multimodal therapy with vaccines, tumor radiation and anti-CD25 mAb using anti-CD4 antibody ascitic fluid (GK1.5 hybridoma), and/or anti-CD8 antibody ascitic fluid (Lyt2.2 hybridoma). The antibody ascitic fluid (10x dilution, 100 μL/dose) was injected into mice on days 5, 6, and 7 after tumor implantation, and the therapy was started. The antibody ascitic fluid was injected every week for the duration of the experiment. The condition of T-cell depletion was validated by flow cytometry using CyChrome-conjugated anti-CD4 mAb and CyChrome-conjugated anti-CD8 (BD Pharmingen); >98% of the relevant cell population was depleted.

**Statistical analysis.** Significant differences were statistically evaluated using ANOVA with repeated measures using Statview 4.1 (Abacus Concepts, Inc., Berkeley, CA). For graphical representation of data, y-axis error bars indicate the SD of the data for each point on the graph.

## Results

**Depletion of CD4+CD25+ Treg cells from mice treated with anti-CD25 mAb.** In previous studies, anti-CD25 mAb has been given over a wide range (250-1,000 μg/mouse) for tumor prevention or the elimination of tumors when given several days before tumor transplant (11, 15, 34). Studies were first conducted here to determine the optimal dose of anti-CD25 mAb to deplete CD4+CD25+ Treg cells from C57BL/6 mice from lymph nodes, peripheral blood, and spleens. Anti-CD25 mAb was given at doses of 75, 150, 300, or 600 μg/mouse (Fig. 1A-C). Five days after administration, inguinal lymph node cells, spleen cells, and peripheral blood cells were analyzed for CD4+CD25+ cells by flow cytometry. The results shown in Fig. 1A-C show that a dose of 300 μg/mouse was optimal for the reduction of CD4+CD25+ cells in lymph nodes, peripheral blood, and spleens. This dose was thus used for the following studies.

Next, we examined the time course of CD4+CD25+ cell depletion after anti-CD25 mAb administration. As seen in Fig. 1D, the percent of CD4+CD25+ cells in lymph nodes greatly decreased 1 day after administration of anti-CD25 mAb and remained decreased until day 28. Virtually identical results were seen in peripheral blood (Fig. 1E). Reduction of CD4+CD25+ cells in spleens followed a similar pattern (Fig. 1F) but was not as pronounced as that seen in lymph nodes or peripheral blood. Mice treated with isotype control antibody showed no depression of CD4+CD25+ cells through the 35-day observation period.

**Determination of optimal timing for anti-CD25 mAb administration in combination with a viral vaccine.** Previous studies have shown that CEA-specific cellular immune responses were enhanced by vaccination with CEA/TRICOM vectors (17, 19). Here, we evaluated whether anti-CD25 mAb administration could enhance these T-cell responses. C57BL/6 mice were s.c. vaccinated on day 0 with rV-CEA/TRICOM, and anti-CD25 mAb was i.p. injected on day −8, −4, 0, 1, 2, 4, or 8, and splenic lymphocytes were evaluated on day 14.

Figure 2A shows CD4+ T-cell proliferation specific for CEA protein. T-cell proliferation in response to CEA peptide was not noted in mice treated with anti-CD25 mAb alone (Δ). Whereas the addition of anti-CD25 mAb on days −8, −4, −2, 0, 1, and 2 enhanced CEA-specific CD4+ responses compared with vaccine alone, optimal responses were clearly observed when the antibody was given at the same time as vaccination (P = 0.0004 versus vaccine alone).

Figure 2B depicts IFN-γ production from CD8+ T cells in response to CEA peptide when mice received vaccines and anti-CD25 mAb at different times. These results also show that administration of the antibody with vaccines at the same time gives optimal CEA-specific responses (50-fold higher than vaccine alone).

**Enhancement of T-cell immune responses specific for self and non–self antigens by vaccines in combination with anti-CD25 mAb.** It has been described that a role for regulatory T cells is to suppress self-reactive immune responses (35). However, a controlled comparison of the effect of elimination of regulatory T cells on the generation of self versus non–self immune responses in the identical host has yet to be investigated. To investigate the relevancy of Treg depletion in a self-antigen system, experiments were conducted using CEA-transgenic mice (20, 21). CEA-transgenic mice were vaccinated with an admixture of rV-CEA/TRICOM (self) and rV-LacZ/TRICOM (nonself) in combination with anti-CD25 mAb. Mice were sacrificed 14 days later, and splenic lymphocytes were evaluated using in vitro assays.

Figure 3A and B shows CD4+ T-cell proliferation specific for CEA or β-gal. CD4+ responses to β-gal (nonself) were, as expected, greater than those to CEA (self). The addition of anti-CD25 mAb enhanced both CEA-specific immune responses (P = 0.002) and β-gal immune responses (P = 0.001 at 6.25 μg/mL protein). These results indicate that anti-CD25 mAb enhanced vaccine-mediated CD4+ responses to a greater extent than the self-antigen than the non–self antigen. We next examined vaccine-induced CD8+ antigen-specific T-cell responses with and without the addition of anti-CD25 mAb (Fig. 3C and D). These results showed, however, a similar
increase in CD8+ responses for self (P = 0.0001) and non–self (P = 0.008 at 1.56 \mu g/mL protein) antigens with the addition of anti-CD25 mAb to vaccines.

**Phenotypic analysis of T cells as a consequence of vaccines, anti-CD25 mAb, and the combination.** Studies were then conducted to determine the phenotype of T cells as a consequence of vaccines, anti-CD25 mAb, and the combination of vaccines and anti-CD25 mAb. As seen in Fig. 4A, CD4+CD25+ cells constituted ~4% to 5% of CD4 cells in spleens. This number was not altered with the administration of vaccines. The addition of anti-CD25 mAb, with or without vaccines, reduced the percent of CD4+CD25+ cells ~10-fold (Fig. 4A). When one examined the CD25+CTLA4+ phenotype, it was seen that vaccines greatly enhanced this phenotype and that the addition of anti-CD25 mAb to the vaccines greatly reduced the presence of these cells (Fig. 4B). An even more striking change in phenotype was seen when one examined the CD25+GITR+ phenotype (Fig. 4C). This phenotype was reduced by the anti-CD25 mAb and enhanced by the administration of vaccines. The addition of anti-CD25 mAb to vaccines, however, greatly reduced this phenotype. We next examined the cytokine production from T cells as a consequence of vaccines, anti-CD25 mAb, or the combination of both. As can be seen in Fig. 4D, the addition of anti-CD25 mAb to vaccines reduced the IL-10 production from CD4+ T cells in response to the CEA antigen by 2.4-fold. This result was even more dramatic when one looks at the reduction in \(\beta\)-gal-specific IL-10 production from CD4+ T cells. The addition of anti-CD25 mAb to vaccines totally eliminated the production of IL-10 in response to \(\beta\)-gal protein in mice vaccinated with a \(\beta\)-gal vaccine. It was interesting to note that the inverse of this was seen when one evaluated IFN production in response to CEA- or \(\beta\)-gal-specific peptides (Fig. 4E). These results clearly show a shift to the Th1 phenotype for both the self and non–self immune responses with the combination of vaccines and anti-CD25 mAb.

**Multimodal therapy of established tumors.** Previous studies have shown that when antiCD25 mAb is given before or 1 day after tumor implantation, antitumor effects can be observed. We confirmed and extended these results in this model system.
When anti-CD25 mAb was given on day 0, 3, or 6 after tumor implantation, tumor growth was inhibited compared with that of the control group ($P < 0.002$; data not shown). However, no antitumor effect of anti-CD25 mAb was seen when mAb was given to mice with established tumors 8 days post-tumor implantation ($P = 0.4$). Based on these studies, further combination therapy studies were conducted 8 days after tumor implantation.

As seen in Fig. 5A and B, administration of vaccines 8 days post-tumor implantation also had no antitumor effects ($P = 0.52$). As seen in Fig. 5C, the addition of anti-CD25 to vaccines also showed no additive antitumor effects ($P = 0.6$ versus control; $P = 0.9$ versus vaccine alone). This was disappointing in light of the demonstration of enhanced CD4$^+$ and CD8$^+$ T-cell responses specific for CEA using this combination therapy.

We have previously reported in two separate studies that the administration of 8 Gy external beam radiation to this tumor shows no antitumor effects (33, 36). We have also previously shown that the use of this dose of radiation up-regulates Fas on tumor cells and facilitates vaccine-mediated killing of tumors (33). These results are confirmed and extended in Fig. 5D ($P = 0.02$ versus vaccine alone). However, it should be noted that whereas significant antitumor effects were seen with vaccines plus tumor radiation, there was no elimination of the 14 established tumors in these experiments. The addition of anti-CD25 mAb to this combination therapy (vaccines plus local radiation of tumors), however, showed significantly greater antitumor effects ($P = 0.01$ versus vaccine plus radiation and $P = 0.0001$ versus vaccine plus anti-CD25 mAb). It should also be emphasized that employing this multimodal therapy of vaccines, local tumor radiation, and anti-CD25 mAb completely eliminated established tumors from 7 of 14 mice (Fig. 5E). Next, we attempted to treat larger tumors with this multimodal regimen by withholding vaccine therapy until day 13 after tumor implantation. When mice were vaccinated s.c. with rV-CEA/TRICOM in combination with anti-CD25 mAb on day 13, irradiated at the site of tumors on day 19, and boosted s.c. with rF-CEA/TRICOM on day 20, tumor growth was again significantly inhibited on day 28 ($P = 0.0001$ compared with the untreated control group). Although no tumors were eliminated in those mice, tumor growth was substantially inhibited in 6 of 10 mice.

Tumor growth was strongly inhibited after radiation in both groups receiving rV-CEA/TRICOM with/without anti-CD25 mAb at priming (Fig. 5D and E). However, tumors were completely eliminated only from the group of mice that received anti-CD25 mAb at the prime vaccination (Fig. 5E). To

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**Fig. 2.** Enhancement of CEA-specific T-cell immune responses induced by rV-CEA/TRICOM in combination with anti-CD25 mAb. C57BL/6 mice were used in these assays (n = 3). Control group was treated with PBS (○). As a mAb monotherapy control, mice were injected i.p. with anti-CD25 mAb (△) on day 0. As a vaccination control, mice were vaccinated s.c. with rV-CEA/TRICOM on day 0 (●). For combination treatment groups, mice were injected i.p. with anti-CD25 mAb on day −8, −4, −2, 0, 1, 2, 4, or 8 in combination with rV-CEA/TRICOM on day 0 (▲). rV-CEA/TRICOM was admixed with rF-GM-CSF. Mice were sacrificed on day 14; splenic lymphocytes were pooled and used for assays. A, CEA-specific CD4$^+$ T-cell proliferation. Cell proliferation was measured by $^3$H-thymidine incorporation. Data is depicted as Δcpm; cell proliferation in response to medium alone was subtracted from that in response to CEA protein (50 μg/mL). SDs are based on the mean of triplicate wells. B, CEA-specific IFN-γ production from CD8$^+$ Tcells. Data is depicted as Δpg/mL; IFN-γ production in response to the control peptide was subtracted from that induced by CEA peptide (10 μg/mL). Dotted line, level of response (●) seen in mice vaccinated with rV-CEA/TRICOM.
examine which effector cells were infiltrating into tumor sites in 0 to 3 days after radiation (days 14-17 after tumor implantation) when tumor mass was drastically reduced, cells were harvested from tumors and analyzed using flow cytometry. The percentage of CD4 T cells (CD3+CD4+), CD8 T cells (CD3+CD8+), natural killer (NK) cells (NK1.1+), dendritic cells (CD11c+MHCII+), B cells (CD19+MHC II+), macrophages (Mac-3+), and neutrophils (Ly-6G+) was compared between two groups (CEA/TRICOM with or without anti-CD25 mAb). We found that CD8 T cells, dendritic cells, and NK cells were increased in tumors in both groups on day 14 (before radiation) compared with those of nonvaccinated mice; there were less of these cells in tumors of mice receiving only vaccine/radiation regimen compared with mice treated with the addition of anti-CD25 mAb. Of particular note, large differences were seen in the percentage of NK cells on days 14 to 17; 5% to 8% in tumors of mice not receiving anti-CD25 mAb versus 20% to 30% in tumors of mice receiving anti-CD25 mAb. An increase of NK cells was not seen in peripheral blood and spleens, and the NK increase seen at tumor sites on days 14 to 17 was not evident after day 22. No differences were seen in terms of the other cell populations. These results indicated that CD8 T cells and NK cells could be associated with antitumor responses induced by the multimodal therapy at local tumor sites in a short time period after tumor radiation.

To determine if immune memory was initiated as a consequence of tumor elimination, mice cured of tumors were rechallenged with MC38-CEA+ tumor cells 30 days after the last vaccination. All mice completely rejected this rechallenge. In addition, we then challenged these mice with CEA-negative parental MC38 tumors; six of seven mice (86%) rejected the parental MC38 tumors. To determine if the antitumor immunity could cross-react to another tumor type, mice were challenged with syngeneic B16 melanoma cells (s.c.) 30 days after the challenge of parental MC38 tumors. None of these mice rejected the B16 tumors; however, tumor growth was significantly suppressed compared with that in the control mice (P = 0.0001). Tumor volume of control mice at day 18 was 1,927 ± 351 mm³, and tumor volume of mice cured after the multimodal therapy was 344 ± 115 mm³. In addition, mice cured after the multimodal therapy in another experiment were challenged with EL-4 lymphoma cells (i.v.) 85 days after the last vaccination. All of those mice remained tumor free for the duration of the experiment (60 days after the EL-4 challenge), whereas all control mice died of tumor on days 20 to 22 after EL-4 implantation. The antitumor effects seen with these CEA-negative tumors indicated that immune responses to other...
antigens commonly expressed on these tumors were induced by the multimodal therapy regimen.

To determine the possible therapeutic mechanisms associated with the multimodal therapy regimen, immunologic analysis was conducted using CEA-transgenic mice implanted s.c. with MC38-CEA+ tumors (Fig. 5E). First, we examined which effector T cells were required for the antitumor activity by depleting CD4 cells and/or CD8 cells. Mice were treated with vaccines, local radiation of tumors, and anti-CD25 mAb by the same schedule described in Fig. 5E. Anti-CD4 antibody and/or anti-CD8 antibody were injected during the therapy as described in the Materials and Methods. In this experiment, tumor growth was significantly inhibited compared with that of the nontreated control group (P = 0.0001), and the tumors were eradicated in 5 of 10 mice (Fig. 5G). When CD4 cells were depleted from mice during the multimodal therapy, tumor reduction after radiation was similar to that of the PBS-treated control group (Fig. 5H, P = 0.67 on day 17). In contrast, when CD8 cells were depleted from mice during the multimodal therapy, tumor reduction was not observed in 8 of 10 mice, and antitumor effects were significantly decreased compared with that seen in the PBS-control group (Fig. 5I, P = 0.02 versus Fig. 5G). When both CD4 and CD8 cells were depleted, antitumor effects were abrogated in all mice, and the antitumor effect was more significantly decreased compared with CD8 cell depletion (Fig. 5J, P = 0.002 versus Fig. 5I). There were no tumor-free mice in all cases when any T-cell subset was depleted (Fig. 5H-I). These results show that CD8 cells were required to induce strong antitumor effects by the multimodal therapy, but that CD4 cells are also needed for complete elimination of tumors.

Next, we examined tumor antigen-specific immune responses of CD4 or CD8 T cells. Mice were treated with vaccines, local radiation of tumors, with or without the addition of anti-CD25 mAb according to the same schedule done in Fig. 5D and E. These mice were sacrificed, and splenocytes were assayed 29 days after tumor implantation. As seen in Fig. 6A, there was a clear increase in the induction of CEA-specific CD4+ T cells with the addition of anti-CD25 mAb to the vaccine/radiation regimen; no immune responses were seen in any of the groups to the human serum albumin control protein. p53 is an antigen known to be overexpressed in the MC38 tumor cell line (30). We thus evaluated immune responses to p53 in light of the fact...
that CEA negative tumors were also eliminated upon rechallenge of mice cured of tumors. As seen in Fig. 6B, T-cell responses to the 15-mer p53 peptide were seen in mice that received vaccines plus radiation. These responses, however, were substantially increased when anti-CD25 mAb was added to the vaccine/radiation regimen \( (P = 0.0017 \text{ at } 0.16 \mu g/mL \text{ protein}) \). This is a clear demonstration of an antigen cascade enhanced by the multimodal therapy regimen.

Moreover, these results were further extended when analyzing CTL activity. As seen in Fig. 6C, CEA-specific CTL activity was greatly enhanced when anti-CD25 mAb was added to the vaccine/radiation regimen. No CTL activity was observed to the VSV control peptide. Only a slight and nonstatistical increase was seen in p53-specific CD8+ T-cell responses with the addition of anti-CD25 mAb (Fig. 6D).

Another endogenous tumor-associated antigen, gp70, has previously been shown to be overexpressed in MC38 tumors (25, 33). As seen in Fig. 6E, there was a strong gp70-specific T-cell response seen in mice receiving vaccines plus local tumor radiation; moreover, there was a statistically significant increase in this gp70 T-cell response \( (P = 0.004 \text{ at } E/T = 5:1) \) in mice treated with the addition of anti-CD25 mAb to the vaccine/radiation regimen. This CTL activity in response to gp70 antigen, not encoded in vaccines but expressed on tumors, was much greater than that seen in response to the other tumor antigens (Fig. 6E versus C and D).

Further studies were conducted to examine the potential role of specific CD8+ T cells directed against antigens not presented by the CEA vaccine, in the antitumor response. Studies were conducted to examine the direct CTL activity against MC38-CEA+ tumor cells (CEA+, p53+, and gp70+), the parental MC38 tumor cells (CEA+, p53+, and gp70+), or B16 tumor cells (CEA+, p53+, and gp70+). We first tested CEA-specific CD8+ T
cells obtained from mice treated with vaccine/radiation regimen with/without anti-CD25 mAb. These cells were stimulated with CEA peptide for 6 days. CEA-specific CD8+ T cells from mice receiving only the vaccine/radiation regimen did not show marked CTL activity against any tumor lines used for assays (Fig. 6F). In contrast, CEA-specific CD8+ T cells from mice receiving the addition of anti-CD25 mAb to the vaccine/radiation regimen showed marked killing activity directed

Fig. 6. Analysis of therapeutic mechanism induced by the multimodal therapy with vaccines, local radiation of tumors and anti-CD25 mAb. CEA-transgenic mice were implanted s.c. with MC38-CEA+ tumors on day 0. These mice were treated with CEA/TRICOM vaccines admixed with rF-GM-CSF, tumor radiation, anti-CD25 mAb, and/or the isotype immunoglobulin according to the same protocol described in Fig. 5. The mice were sacrificed; splenic lymphocytes were pooled and used for in vitro assays 29 days after tumor implantation (n = 4). A and B: tumor antigen-specific CD4+ T-cell proliferation. Open squares, control response to human serum albumin (HSA, 50 μg/mL) in the group vaccinated with CEA/TRICOM vaccines in combination with radiation and isotype immunoglobulin. Diamonds, control response to HSA in the group vaccinated with CEA/TRICOM vaccines in combination with radiation and anti-CD25 mAb. Data is depicted as Δcpm: negative control responses were subtracted from that in response to CEA protein or p53 class II peptide. SDs are based on the mean of triplicate wells. C-E: CTL activity in response to tumor antigen peptide. C: CEA-specific CTL activity (E/T ratio = 50:1). D: p53-specific CTL activity (2 μg/mL p53 peptide). E: gp70-specific CTL activity (1 μg/mL gp70 peptide). Asterisks, mean 51Cr release in response to the control peptide (control response, range 4.95-5.51% cpmp, P > 0.05 among groups). Data is depicted as Δ%; negative control responses were subtracted from that induced by each antigen peptide. SDs are based on the mean of triplicate wells. Closed squares, mice treated with vaccines in combination with tumor radiation and isotype immunoglobulin. Crossed squares, mice treated with vaccines in combination with tumor radiation and anti-CD25 mAb. F-H: direct CTL activity against tumor cells (E/T ratio = 50:1). F: CTL activity of CEA-specific CTLs (CEA-CTLs). Splenic lymphocytes were stimulated with CEA peptide for 6 days and used for 51Cr release assay. G: CTL activity of p53-specific CTLs (p53-CTLs). Splenic lymphocytes were stimulated with p53 peptide for 6 days and used for 51Cr release assay. H: CTL activity of gp70-specific CTLs (gp70-CTLs). Splenic lymphocytes were stimulated with gp70 peptide for 6 days and used for 51Cr release assay. I: generation of gp70-specific CD8+ T cells by the multimodal therapy with vaccines, tumor radiation, and anti-CD25 mAb. Cells from tumors, blood, and spleens were stained with anti-CD3 mAb, anti-CD8 mAb, and gp70-tetramer. a-c, mice were vaccinated with vaccines in combination with tumor radiation and isotype immunoglobulin. a, tumor-infiltrating T cells. b, peripheral blood T cells. c, splenic T cells. d-f, mice were vaccinated with vaccines in combination with tumor radiation and anti-CD25 mAb, tumor-infiltrating T cells. e, peripheral blood T cells. f, splenic T cells. The numbers indicate the percentage of gp70-tetramer-binding CD8+ T cells in total cells. These panels were depicted after gating the CD3+ fraction.
against MC38-CEA+ tumor cells (Fig. 6F). The CTL activity was significantly higher than that seen in mice treated with only vaccine/radiation (Fig. 6F, P = 0.0001). When CEA-negative MC38 cells or B16 cells were used as a tumor targets, however, CTL activity was not observed, and there were no significant differences between these groups (Fig. 6F). Next, p53-specific CD8+ T cells, which were stimulated with p53 peptide for 6 days, were tested for direct CTL activity against tumors. P53-specific CD8+ T cells from mice treated with only vaccine/radiation did not exhibit CTL activity against any tumor lines used for assays (Fig. 6G). However, p53-specific CD8+ T cells from mice receiving the addition of anti-CD25 mAb to the vaccine/radiation showed marked killing activity directed against all tumor lines, and there were significant differences compared with those seen in mice receiving only vaccine/radiation (Fig. 6G, P < 0.0004). gp70-specific CD8+ T cells from mice treated with the vaccine/radiation regimen showed slight CTL activity against MC38-CEA+ and MC38 tumor cells but not against B16 (Fig. 6H). In contrast, gp70-specific CD8+ T cells from mice receiving the addition of anti-CD25 mAb to the vaccine/radiation regimen showed killing activities directed against all tumor lines, and significant differences were observed compared with those of mice receiving only vaccine/radiation (Fig. 6H, P < 0.0004). Interestingly, the CTL activity shown by gp70-specific CTLs was the greatest in this group compared with those shown by the other CTLs (Fig. 6I versus F and G).

Because gp70-specific tetramer was available, we conducted gp70-tetramer binding assays to detect the gp70-specific CD8+ T cells in these mice. It should be pointed out that CEA- and p53-specific tetramer was not available, but it is clearly shown (Fig. 6A–H) that CEA- and p53-specific T cells are also present in the periphery after vaccination. Cells were harvested from tumors, peripheral blood, and spleens of these mice and analyzed for gp70-tetramer binding using flow cytometry. As seen in Fig. 6A, 15% of CD8+ T cells seen in tumors were gp70 specific following treatment of mice with vaccines and local tumor radiation. This number was increased to 35% when mice were treated with the multimodal therapy using vaccines, radiation, and anti-CD25 mAb (Fig. 6Id). Similar increases in gp70-specific CD8+ T cells were also seen in blood and spleens from mice receiving the multimodal therapy compared with those from mice receiving only vaccines and radiation (Fig. 6le and f).

These studies thus showed that a multimodal therapy was required for the elimination of established tumors and that an increase in CD4+ and CD8+ cells specific for a vaccine-directed antigen (CEA) as well as an antigen cascade of T-cell responses to other tumor-associated antigens (p53 and gp70) was associated with antitumor effects.

**Discussion**

Previous studies have shown that in vitro depletion of CD4+CD25+ immunosuppressive Treg cells can enhance T-cell immune responses (3, 37–53). In addition, it has been also shown that in vivo antitumor activity could be augmented by depletion of CD4+CD25+ cells via anti-CD25 mAb administration (10, 11, 13, 14, 34). Based on the emerging characteristics of the suppressive actions of CD4+CD25+ cells, many research-
by the induction of an antigen cascade of immune responses to other antigens commonly expressed on these tumors. Many studies have shown antitumor efficacy of anti-CD25 mAb itself (10, 11, 13, 14, 34). In these studies, however, anti-CD25 mAb was given several days before or immediately after tumor implantation. In the tumor model employed here, the administration of anti-CD25 mAb to mice with well-established tumors 8 days after tumor implantation was ineffective (Fig. 5C). The administration of vaccines 8 days after tumor implantation was also ineffective (Fig. 5B). We have also previously shown that the single treatment with external beam radiation of established tumors is also insufficient to cause antitumor effects (33, 36). These studies did show, however, that external beam radiation of tumors at doses below that which cause antitumor effects up-regulated the expression of Fas on tumor cells and thus rendered them more susceptible to vaccine-induced T-cell mediated lysis. It was further shown that the up-regulation of Fas was necessary for tumor killing by the fact that a tumor expressing a dominant-negative Fas was insensitive to killing. It was also shown in these studies that both CEA expression in the tumor and the use of a vaccine expressing the CEA transgene were both necessary for antitumor effects (33).

The studies reported here show the complexity of achieving a cure of a well-established tumor. Here we have employed three different modalities to achieve this goal: (i) vaccines to induce antigen-specific T-cell responses, (ii) external beam radiation of tumors to up-regulate Fas and thus make tumor cells more susceptible to T-cell killing, and (iii) the use of anti-CD25 mAb to eliminate CD4+CD25+ suppressor cells. Recent in vitro studies have shown that external beam radiation of a range of tumor types at doses below cytotoxic doses will up-regulate Fas and render human tumor cells more susceptible to antigen-specific T-cell killing (54). Several vaccine trials employing TRICOM vaccines are also in progress (55), and external beam radiation of tumors is a well-established modality for the treatment and/or palliation of a range of human tumors. The use of several drugs, antibodies, or fusion proteins to reduce or eliminate human suppressor cells is also currently being evaluated in the clinic. The studies reported here thus form the rationale for potential clinical trials employing multimodal immune mediated therapies.

References


