Eradication of Large Tumors in Mice by a Tritherapy Targeting the Innate, Adaptive, and Regulatory Components of the Immune System

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Abstract

Targeting the human papillomavirus (HPV) E7 antigen to dendritic cells with the adenylate cyclase (CyaA) of *Bordetella pertussis* as a vaccine vector led to potent therapeutic immune responses against TC-1 tumors in a murine model of cervical carcinoma induced by HPV. However, as the time between tumor graft and vaccination increased, the antitumor efficacy of the CyaA-E7 vaccine gradually decreased. The vaccine had no effect if the tumor diameter was >8 mm. Analyses of regulatory cells recruited during TC-1 tumor growth revealed a high number of splenic MDSCs and a large percentage of regulatory T cells, particularly in the tumor. Administration of a tritherapy including CpG complexed with a cationic lipid, low-dose cyclophosphamide, and the CyaA-E7 vaccine completely overcame tumor-associated immunosuppression and eradicated large, established tumors in almost all treated animals. This strong antitumor response was followed by a large expansion of regulatory T cells in tumor, spleen, and tumor-draining lymph nodes and of splenic neutrophils. These findings indicate that immunotherapeutic strategies that simultaneously target innate, adaptive, and regulatory components of the immune system are effective in the eradication of large tumors. [Cancer Res 2007;67(18):8847–55]

Introduction

Successful implementation of immunotherapeutic approaches developed in murine tumor models for human malignancies remains a major challenge, despite intense efforts to improve the two main components of classic vaccines (i.e., the antigen delivery system and the adjuvant).

We have developed a new vaccine vector based on the adenylate cyclase (CyaA) of *Bordetella pertussis* that improves antigen delivery by targeting antigen to the dendritic cells. CyaA binds specifically to the $\alpha_M\beta_2$ integrin (CD11b/CD18; ref. 1) and delivers its catalytic domain into the cytosol of $CD11b^+$ cells (2, 3). Thus, $CD4^+$ and $CD8^+$ T-cell epitopes inserted into the catalytic site of CyaA are processed and presented by MHC class II and I molecules, respectively, at the surface of dendritic cells (4). We have reported previously that recombinant CyaA carrying a $CD8^+$ T-cell epitope from ovalbumin induces strong therapeutic immunity in mice against tumors expressing this antigen (5).

After breast cancer, cervical carcinoma is the second most common cancer in women, worldwide, and the fifth most frequent cancer overall, with an estimated prevalence of 1.4 million cases (6). There is now consistent evidence that chronic infection of the genital tract by various mucosatropic human papillomavirus (HPV) types (7) cause cervical cancer. Expression of HPV oncogenic proteins E6 and E7 are required for onset (8) and maintenance of malignant transformation (9). Cellular immunity to E7 has been associated with clinical and cytologic resolution of HPV-induced lesions (10). Thus, HPV-neoplasia is a good target for the development of immunotherapeutic strategies. Recently, we have shown that recombinant CyaA carrying a modified form of the HPV type 16-E7 protein (CyaA-E7) induces the eradication of palpable tumors generated by s.c. grafts of E6/E7–expressing TC-1 cells, a validated model for testing the efficacy of immunotherapeutics against HPV-associated neoplasia (11). This therapeutic effect was shown by injection of the vaccine, CyaA-E7, to mice 10 days after the graft of tumor cells. Although tumors were already detectable at the time of treatment by the vaccine, this experimental model may, however, not reflect human situation. In the present study, we indeed show that the therapeutic efficacy of the vaccine is progressively lost as the tumor grows, reaching a nonsignificant value if the vaccination is done 25 to 30 days after the tumor graft.

Currently available anticancer vaccine candidates might be improved by stimulation of innate immunity, in particular through Toll-like receptors (TLR), the natural sensors for infection. Although all members of the TLR family share certain structural and functional properties, the signals delivered by various TLRs may elicit qualitatively and quantitatively different immune responses (12). Also, these vaccine candidates can be combined with chemotherapeutic compounds. Chemotherapy enhances antitumor immunity by reducing tumor burden, increasing tumor cell sensitivity to CTL (13), and killing/inactivating immunoregulatory cells (14).

In the present study, we thus investigate how well various treatments enhanced the antitumor immunity induced by CyaA-E7 in a tumor setting in which this vaccine could not control the growth of large, established tumors. We show that a tritherapy that simultaneously targets innate (with TLR9 ligand), adaptive (with CyaA-E7), and regulatory (with low-dose cyclophosphamide) components of the immune system eradicates large tumors (tumor diameter of ~8 mm) in almost all treated animals.

Materials and Methods

Mice and tumors. Specific pathogen-free 5-week-old female C57BL/6 mice were purchased from Charles River and were kept in the Pasteur

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

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Institute animal facilities under pathogen-free conditions with water and food *ad libitum*. C57BL/6-RAG1$^{-/-}$ mice were kindly donated by Dr. Ignacio Melero (Centro de Investigación Médica Aplicada, Pamplona, Spain). Experiments involving animals were conducted according to the institutional guidelines for animal care.

TC-1, tumor cells expressing HPV16-E6 and HPV16-E7 proteins derived from primary mouse lung epithelial cells, were obtained from the American Type Culture Collection (LGC Promochem; ref. 15). EL4-E7 cells, a mouse lymphoma expressing HPV16-E7, were a kind gift from I.H. Frazer (University of Queensland, Queensland, Australia; ref. 16). Cells were maintained in RPMI 1640 with GlutaMAX supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.4 mg/mL geneticin, and 5×10^{-5} mol/L 2-mercaptoethanol (Life Technologies).

Tumor cell lines (5×10^5 TC-1 cells or 4×10^6 EL4-E7 cells) were injected into the shaved back (left side) of C57BL/6 mice in 200 μL PBS. Tumor size, presented as the average of two perpendicular diameters (millimeters), was measured at regular intervals.

Reagents. The synthetic peptide $E7_{49-57}$ (RAHYNIVTF, one-letter code for amino acid) corresponding to the HPV16-E7 H2-D^b–restricted epitope (17) was purchased from NeoMPS.

The detoxified form of the CyaA of *B. pertussis* carrying a truncated form of the E7 protein (CyaA-E7) and the control detoxified CyaA without any insert (CyaA) were prepared as described previously (11).

CpG oligodeoxynucleotides (type A, CpG 2216: 5′-**GGG**GGACCGATCGTC**GGGGGG**-3′; type B, CpG 1826: 5′-**TCCATGACGTTCCTGACGTT**-3′) were synthesized by Proligo. Boldface nucleotides correspond to the phosphorothioate backbone. Polyuridine was purchased from Sigma; R848 was from PharmaTech; and polyinosinic–poly-CMP (PIC) was from Invivogen. Thirty micrograms CpG oligodeoxynucleotides or polyuridine were diluted in 50 μL Opti-MEM medium (Life Technologies) and mixed with 60 μg *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate (DOTAP; Roche) diluted in 100 μL Opti-MEM. Other TLR ligands were diluted in PBS before injection. Cyclophosphamide (Sigma) was diluted in PBS, and doxorubicin (Calbiochem) was diluted in sterile water before injection.

Other than chemotherapeutic agents that were given 24 h before vaccine, the various antigenic formulations and adjuvants were injected simultaneously. I.v. administration was done by retroorbital injection in a volume of 200 μL and intratumoral (i.t.) administration was done by injection in a volume of 50 μL.

Flow cytometry analysis. Solid tumors, spleens, and inguinal lymph nodes were excised, ground to prepare cell suspensions, and subsequently stained to analyze cell changes during tumor growth and after therapy. Monoclonal antibodies used for staining were FITC-conjugated anti-CD11b, anti-CD4, anti-CD69, anti-Ly6G, and anti-CD8; phycoerythrin-conjugated anti-GR1, anti-CD124, and anti-NK1.1; and allophycocyanin-conjugated anti-CD25, anti-CD44, anti-CD69, anti-CD8, anti-CD11b, and anti-CD11c (all from PharMingen). Phycoerythrin-conjugated H-2D^b/$E7_{49-57}$ tetramers were obtained from Beckman Coulter. Regulatory T cells (Treg) were stained with the Mouse Regulatory Staining kit (E-Biosciences).

FACScalibur (Becton Dickinson) was used for flow cytometry and events were analyzed with CellQuest software (Becton Dickinson).

***In vivo* killing assay.** Naïve spleen cells were pulsed for 30 min with 9 μmol/L $E7_{49-57}$ peptide at 37°C. After extensive washing, cells were labeled with 2.5 μmol/L carboxyfluoroscein succinimidyl ester (CFSE^{high}; Molecular Probes). Control nonpeptide–treated splenocytes were labeled with 0.25 μmol/L CFSE (CFSE^{low}). CFSE^{high} and CFSE^{low} cells were mixed in a 1:1 ratio and 10^7 cells were injected i.v. into naïve or immunized animals. Twenty-four hours later, spleens were removed and single-cell suspensions were analyzed by flow cytometry to determine the ratio of CFSE^{high} to CFSE^{low} cells. The percentage of specific lysis was calculated as follows: percent specific lysis = 100 − [100 × (% CFSE^{high} immunized /% CFSE^{low} immunized) / (% CFSE^{high} control / % CFSE^{low} control)].

Anti-CD3 immunohistochemistry. Tumor samples were fixed in formalin and embedded in paraffin. Three-micrometer sections were microwaved for 10 min in Tris-EDTA (0.001 mol/L; pH 9) for antigen retrieval and endogenous peroxidase was quenched with Peroxidase Blocking Reagent (DAKO). Tissue sections were incubated overnight at 4°C with affinity-purified anti-human CD3 (LabVision Corporation) diluted 1:300 in Tris-buffered saline. The peroxidase activity was revealed using anti-rabbit EnVision System (Dako) and 3,3′-diaminobenzidine + Substrate Chromogen System (DAKO). Finally, sections were counterstained with methyl green.

Statistical analysis. Monolix software[5] was used for analysis of tumor growth data with nonlinear mixed effect models. Mean diameters of tumors over time were fitted using the model described previously (18) and treatments were compared using the likelihood ratio test. Kaplan-Meier plots were used to analyze survival. Prism software (GraphPad Software, Inc.) was used to determine the significance of differences in survival curves with a log-rank test. Data from *in vivo* CTL and tetramer staining were compared by ANOVA followed by Dunnett's posttest. P values <0.05 were considered to be statistically significant.

Results

The CyaA-E7 therapeutic effect is abrogated in mice with large tumors. We have shown previously that a single injection of the CyaA recombinant protein carrying the HPV-E7 antigen (CyaA-E7) 10 days after injection of 5×10^4 TC-1 tumor cells induces complete regression of tumor growth and leads to survival of all treated mice (11). In this study, we injected mice with 5×10^5 TC-1 cells and at various time points administered a single i.v. injection of 50 μg CyaA-E7 to determine whether such therapeutic vaccination is efficient at later stages of tumor growth. After an initial phase of tumor growth, tumors were rejected in all mice treated 4 days after the injection of TC-1 cells. Tumor relapse occurred in one mouse at day 60 (Fig. 1*A*). The antitumor efficacy of the CyaA-E7 vaccine gradually decreased with increasing time between TC-1 tumor graft and administration of CyaA-E7 (i.e., with increasing tumor size). Thus, CyaA-E7 administered 25 days after the injection of TC-1 cells protected only 20% of mice from tumor growth. Despite this, tumor growth was slower and survival rates were higher than in PBS-treated mice ($P < 0.0001$ for both tumor growth and survival rates; Fig. 1*A* and *B*). In contrast, mice treated 30 days after tumor cell injection had tumor growth ($P = 0.75$) and survival rates ($P = 0.9337$) similar to those observed for the PBS-treated group (Fig. 1*A* and *B*). Thus, CyaA-E7 administered 1 month after tumor cell injection did not induce therapeutic antitumor responses. This may be similar to the generally observed failure of therapeutic vaccines in patients with advanced tumors.

TLR ligands and cyclophosphamide enhance the therapeutic efficacy of CyaA-E7 on advanced tumors. We then tested whether adjuvants can restore the antitumor responses induced by CyaA-E7 administration in mice suffering from advanced tumors. For these experiments, we treated mice 25 days after tumor cells were injected because this was the latest time point that CyaA-E7 therapeutic activity was detected.

Recently, TLR ligands have received a lot of attention because they trigger dendritic cell maturation *in vivo*. Thus, we selected five synthetic TLR ligands: a TLR-3 ligand, PIC; two TLR-7 ligands, polyuridine and R848; and two TLR-9 ligands, CpG-A and CpG-B. We complexed CpGs and polyuridine with the cationic lipid DOTAP to protect them from degradation and facilitate their uptake.

A significant increase in tumor regression was observed in mice given with CyaA-E7 and polyuridine, CpGs, or PIC 25 days after the

[5] http://www.monolix.org/

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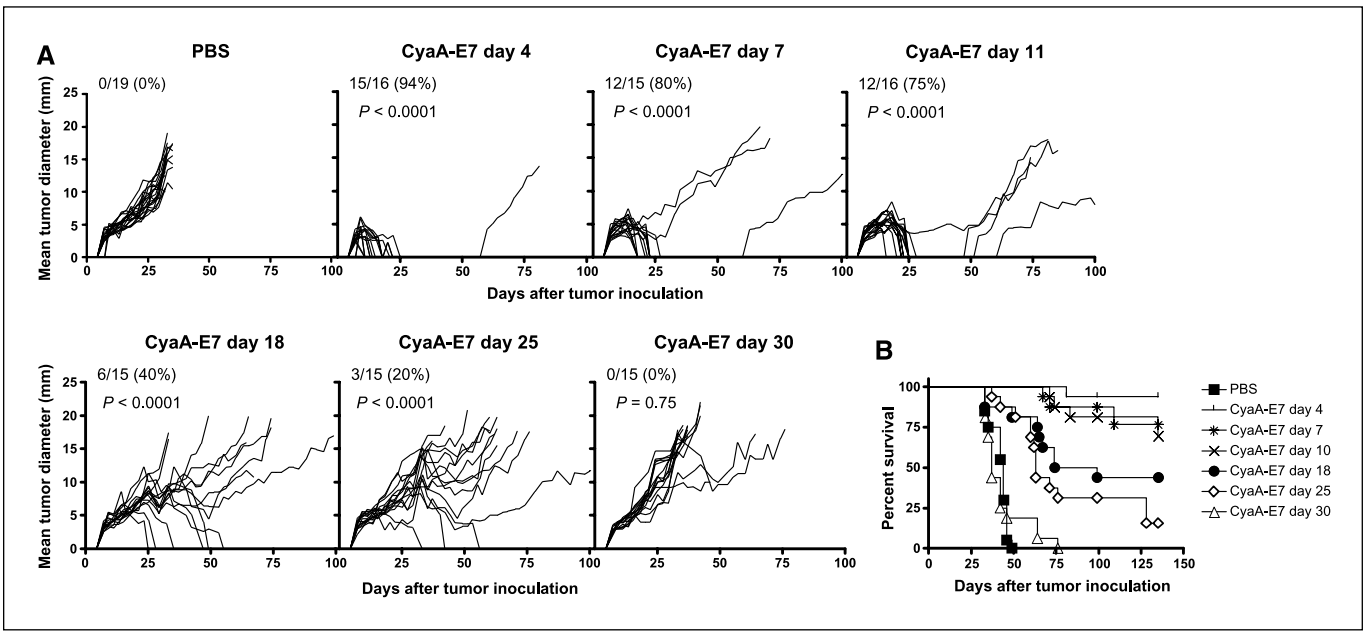

Figure 1. Abrogation of CyaA-E7 therapeutic effects in mice with large tumors. *A,* C57BL/6 mice were injected with 5×10^5 TC-1 cells on day 0 and then received PBS on day 4 or were treated i.v. with 50 μg CyaA-E7 on day 4, 7, 11, 18, 25, or 30. Each curve represents the mean tumor diameter in a single mouse. The number of tumor-free mice on day 135 relative to the total number of animals included, the percentage surviving on day 135, and the *P* value determined by the likelihood ratio test comparing tumor growth in groups of treated mice and tumor growth in the PBS-treated group are shown. *B,* Kaplan-Meier plot of mouse survival. The groups were compared with the PBS-treated group with the log-rank test. Survival rates in groups treated with Cya-E7 on day 4, 7, 11, 18, or 25 were significantly different from mice treated with PBS ($P < 0.0001$). Mice treated with CyaA-E7 on day 30 had similar survival rates as PBS-treated mice ($P = 0.93$). Mice were killed when tumor diameter reached 20 mm or when necessary due to the sanitary status of the animals. Pooled data of two independent experiments.

injection of TC-1 cells. We did not observe this result with R848 administration. Survival percentages ranged from 41% with polyuridine and PIC to 50% to 58% with CpG-B and CpG-A (Fig. 2*A* and *B*). The tumors continued to grow for 5 to 7 days after injection of CyaA-E7 with these TLR agonists, reaching diameters of up to 14 mm. Then, most of the tumors began to shrink, reflecting a successful induction of a therapeutic immune response. In various mice, this phase lasted 25 days, ultimately leading to the complete cure of the tumor. However, before the tumors could be eradicated, tumor relapse occurred in some mice.

We tested the therapeutic effect of two of these TLR ligands (PIC and CpG-B) administered alone, without the CyaA-E7 vaccine, to study whether these TLR ligands could enhance the immune response primed by the tumor. None of these TLR ligands administered alone affected tumor growth (Fig. 2*A*). Therefore, immune responses elicited by the CyaA-E7 protein are required to achieve regression of these large tumors.

There is increasing evidence that immunomodulating doses of chemotherapeutic agents can overcome immune tolerance of tumor-bearing mice, favoring antigen-specific immune responses. Thus, we analyzed whether chemotherapeutic agents could also restore the therapeutic activity of CyaA-E7 when administered to mice with large tumors. We selected two drugs for this purpose. One was cyclophosphamide, an alkylating agent of the nitrogen mustard type that enhances the efficacy of antitumor vaccines (19, 20). The other was doxorubicin, an intercalating agent that inhibits the action of the enzyme topoisomerase II. After i.t. injection, this agent induces immunogenic cell death, controlling tumor growth (21). In our large-tumor model, doxorubicin did not enhance the effect of CyaA-E7 if given i.t. (Fig. 2*A*) or i.v. (data not shown). In contrast, mice injected with a low dose of cyclophosphamide 24 h before the CyaA-E7 vaccine had significantly slower tumor growth than control mice. Also, such treatment eradicated the tumors in >50% of the animals. Unlike the TLR ligands, cyclophosphamide alone significantly delayed tumor growth relative to that observed in PBS-treated mice ($P = 0.0002$), but this effect was transient and was followed by tumor relapse (Fig. 2*A*). This tumor growth delay was not observed in RAG1$^{-/-}$ mice (Supplementary Fig. S1), strongly suggesting that this effect was mediated by T cells. Finally, the antitumor efficacy of the combined administration of cyclophosphamide and CyaA-E7 was not improved by a 3-fold cyclophosphamide dose increase (Supplementary Fig. S2). Cyclophosphamide at 100 mg/kg displayed maximal antitumor efficacy, reducing the possible side effects associated to this combined therapy.

Eradication of large tumors by a tritherapeutic treatment combining chemotherapy, a TLR-9 ligand, and an antitumor vaccine. We hypothesized that as CyaA-E7, cyclophosphamide, and CpG have different mechanisms of action, they would have a synergic effect. Thus, we administered cyclophosphamide to mice 24 days after injection with TC-1 cells. Twenty-four hours later, mice were injected with CyaA-E7 and CpG-B in DOTAP. This tritherapy had a strong antitumor effect with a survival rate of 87.5%. The tumor was not eradicated in only 2 of 16 mice receiving the tritherapy (Fig. 3*A* and *B*). The CyaA-E7 vaccine was essential for this therapeutic effect. Administration of cyclophosphamide and CpG-B/DOTAP did not induce tumor regression although mice receiving this treatment had significantly slower tumor growth than PBS-treated mice ($P < 0.0001$), as observed previously after administration of cyclophosphamide alone (Fig. 3*A*).

We administered the tritherapy to tumor-bearing mice 25, 30, and 40 days after tumor cell injection to find the therapeutic limits of this treatment. The antitumor efficacy of the tritherapy was smaller if administered on day 30 than on day 25. Despite this,

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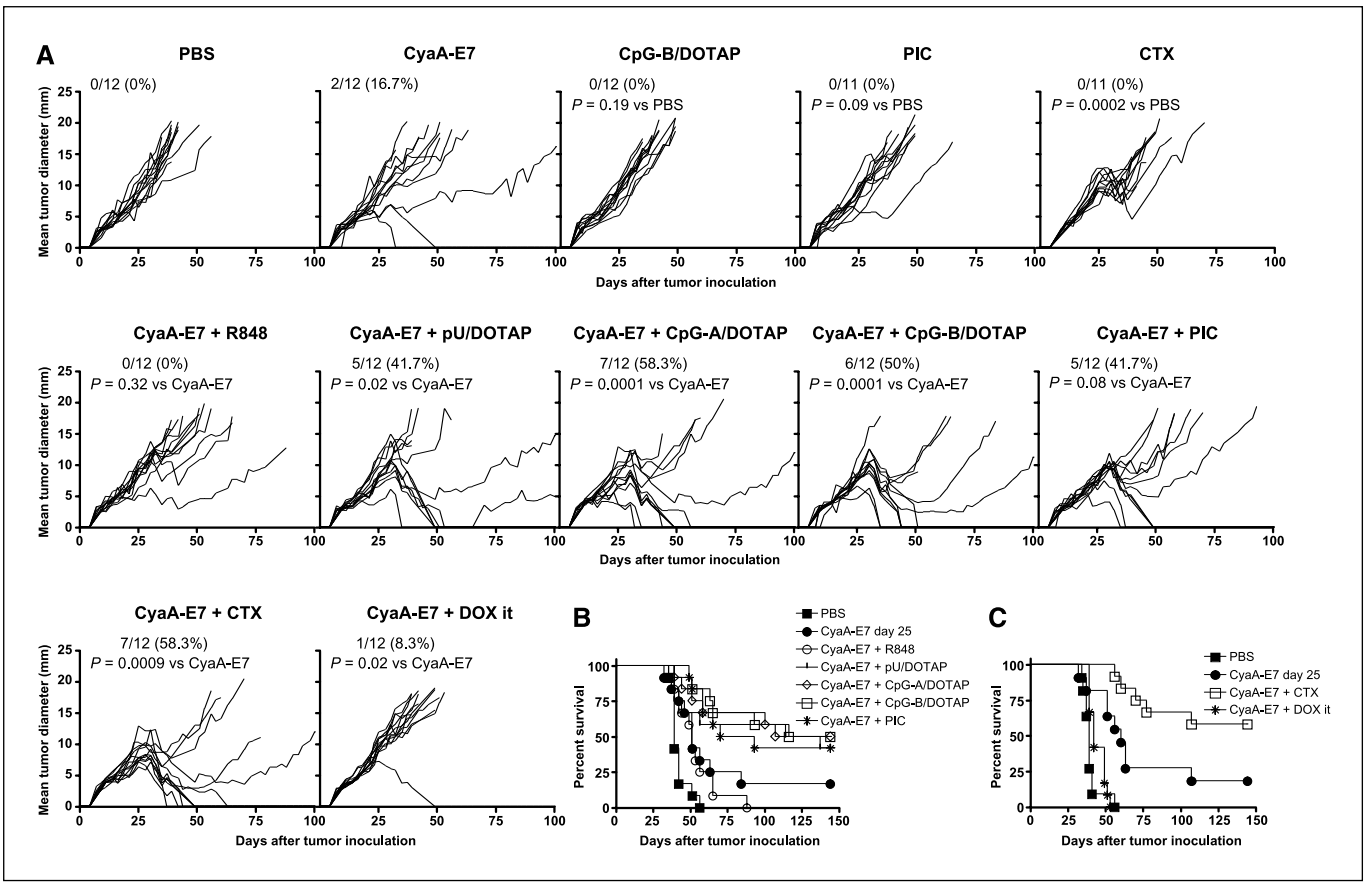

Figure 2. Combined tumor treatment with CyaA-E7 and various TLR ligands or chemotherapy agents. *A,* C57BL/6 mice were injected on day 0 with 5×10^5 TC-1 cells. On day 24, chemotherapy agents were administered whereas vaccine and TLR ligands were injected on day 25. Groups of mice received PBS, 50 μg CyaA-E7 alone, 30 μg CpG-B complexed with 60 μg DOTAP, 25 μg PIC, or 2.5 mg cyclophosphamide (*CTX*) i.p.; 50 μg CyaA-E7 combined with 50 μg of R848, 30 μg polyuridine (*pU*)/DOTAP, CpG-A/DOTAP, CpG-B/DOTAP, 25 μg of PIC, 2.5 mg cyclophosphamide i.p, or 125 μg doxorubicin (*DOX*) i.t. Each curve represents the mean tumor diameter in a single mouse. The number of tumor-free mice on day 150 relative to the total number of animals included, the percent surviving on day 150, and the *P* value determined by the likelihood ratio test comparing tumor growth in various treated groups with tumor growth in the PBS-treated group and CyaA-E7–treated group are indicated for each set of experiments. *B,* Kaplan-Meier plot of mouse survival with TLR ligands. Various groups were compared with CyaA-treated mice with the log-rank test: CyaA-E7 + R848 ($P = 0.44$), CyaA-E7 + polyuridine/DOTAP ($P = 0.17$), CyaA-E7 + CpG-A/DOTAP ($P = 0.048$), CyaA-E7 + CpG-B/DOTAP ($P = 0.027$), and CyaA-E7 + PIC ($P = 0.057$). *C,* Kaplan-Meier plot of mouse survival with chemotherapy agents. Various groups were compared with CyaA-E7–treated mice with the log-rank test: CyaA-E7 + cyclophosphamide ($P = 0.018$) and CyaA-E7 + doxorubicin i.t. ($P = 0.0016$). Mice were killed when tumor diameter reached 20 mm or when necessary due to the sanitary status of the animals. Pooled data of two independent experiments.

tumors were eradicated in 41.7% of the animals (Fig. 3*A* and *B*). Even if tritherapy was given on day 40, when tumor diameter ranged between 14 and 19 mm, tumors were eradicated in two mice. Tumors started to regress in all treated mice, reflecting the induction of an effective immune response that lasted ~20 days. This regression phase was then followed by tumor relapse in most animals (Fig. 3*A* and *B*). However, the regression phase could be prolonged by a second administration of the tritherapy at day 55, leading to tumor eradication in 43% of treated mice (Supplementary Fig. S3).

We then injected mice s.c. with transfected EL4 cells that produce E7 protein (EL4-E7 cells) to expand this study with another tumor model (16). These cells grew faster than TC-1 cells and by day 14, EL4-E7 tumors were similar in size to those of TC-1 tumors on day 30 (Supplementary Fig. S4). We administered the tritherapy to mice 7, 14, or 21 days after tumor cell injection. Tritherapy eliminated the tumor if administered up to 14 days after tumor cell injection, but did not do so if given 21 days after tumor cell injection. Similarly to the TC-1 model, all tumors started to regress. However, most of them relapsed.

Dynamics of effector and regulatory cells during tumor development. We then investigated the mechanisms that may account for the loss of therapeutic activity of CyaA-E7 in mice bearing large tumors. In particular, we studied whether expansion of Treg and/or myeloid-derived suppressor cells (MDSC) were responsible for impaired CyaA-E7 activity. We analyzed the percentages of these cell populations in the spleen, inguinal draining lymph nodes and tumor at various time points after the injection of TC-1 cells (Fig. 4).

We found high percentages of $CD4^+CD25^+FoxP3^+$ cells infiltrating the tumor at all time points analyzed, indicating that these cells were specifically recruited and/or expanded (Fig. 4). This population also increased over time in spleens and draining lymph nodes of tumor-bearing mice. We observed this in the draining lymph node by day 10: Whereas this population made up 8.7% of the population before tumor injection, it made up 10.8% of the population 10 days later ($P < 0.05$), 12.6% by day 25 ($P < 0.01$), and 16.1% by day 40 ($P < 0.01$). In the spleen, we observed a significant difference only on day 40 when these cells made up 20.7% of the population rather than the 11.0% observed before tumor cell injection ($P < 0.01$).

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A myeloid-derived CD11b$^+$GR1$^+$ cell population increased over time in the spleen of tumor-bearing animals. On day 40, there was a significantly higher percentage of these cells in the spleens of tumor-bearing mice (11.6%) than in control mice (1.0%, $P < 0.01$). This population expressed the interleukin 4 receptor α chain (CD124), recently associated with immunosuppressive MDSCs (Supplementary Fig. S5*A*; ref. 22). Thus, Treg expansion inside the tumor was substantial and occurred, to lesser extent, in the spleen and lymph nodes. We detected the expansion of MDSCs only in spleens of mice with late-stage tumors.

Analysis of antitumor immune responses induced by tritherapy in control and tumor-bearing mice. We analyzed the CD8$^+$ T-cell response specific for the E7$_{49-57}$ CD8$^+$ T-cell epitope by determining the percentage of tetramer$^+$ cells and the *in vivo* cytolytic activity generated in control and tumor-bearing mice to study the immune responses induced by the various treatments. In control mice, there were significantly more tetramer$^+$ cells after immunization with CpG-B/DOTAP + CyaA-E7 than with CyaA-E7 alone, independent of the administration of cyclophosphamide. The *in vivo* CTL assay indicated that there was significantly more cytolytic activity after immunization with CyaA-E7 + CpG-B/DOTAP or tritherapy (Fig. 5).

Mice injected with TC-1 tumor cells 25 days before immunization had larger percentages of tetramer$^+$ cells after both treatments. However, none of the tested combinations was significantly different from immunization with CyaA-E7 alone because of high interindividual variability (Fig. 5). However, we observed a significantly stronger *in vivo* CTL activity in mice immunized with CyaA-E7 + CpG-B/DOTAP in the absence or presence of cyclophosphamide ($P < 0.05$) than with CyaA-E7 alone (Fig. 5). The administration of cyclophosphamide with CyaA-E7 did not enhance the immune response induced by CyaA-E7, despite its strong antitumor activity.

No tetramer$^+$ cells were detected in tumor-bearing mice 40 days after TC-1 injection. The *in vivo* cytolytic activity was low but still detected (Fig. 5). Thus, the expansion of MDSCs and Treg associated with TC-1 growth gradually impairs the antitumor immune response induced by tritherapy.

Dynamics of effector and regulatory cells after tritherapy on day 25. We evaluated the changes in the immune system induced by tritherapy during tumor rejection. Specifically, we analyzed changes in Treg, MDSCs, tumor-specific CD8$^+$ T cells, and NK cells.

Over time, we observed fewer CD4$^+$CD25$^+$FoxP3$^+$ cells in spleen and draining lymph node. This decrease was slightly more pronounced than that for total CD4$^+$ cells (Fig. 6). We observed the largest difference in tumor-associated Treg: Whereas 50% of CD4$^+$ cells were CD25$^+$FoxP3$^+$ in nontreated mice, 30% were CD25$^+$FoxP3$^+$ 4 and 7 days after treatment (Fig. 6). However, 11 days after tritherapy, there were higher percentages of CD25$^-$FoxP3$^+$ cells in all organs analyzed, leading to significantly higher percentages of CD4$^+$FoxP3$^+$ T cells in the spleen and draining lymph node and to recovery of initial percentages of CD4$^+$FoxP3$^+$ T cells in the tumors (Fig. 6). Paralleling the expansion of FoxP3$^+$ cells, there were substantially more tumor-specific CD8$^+$ T cells in

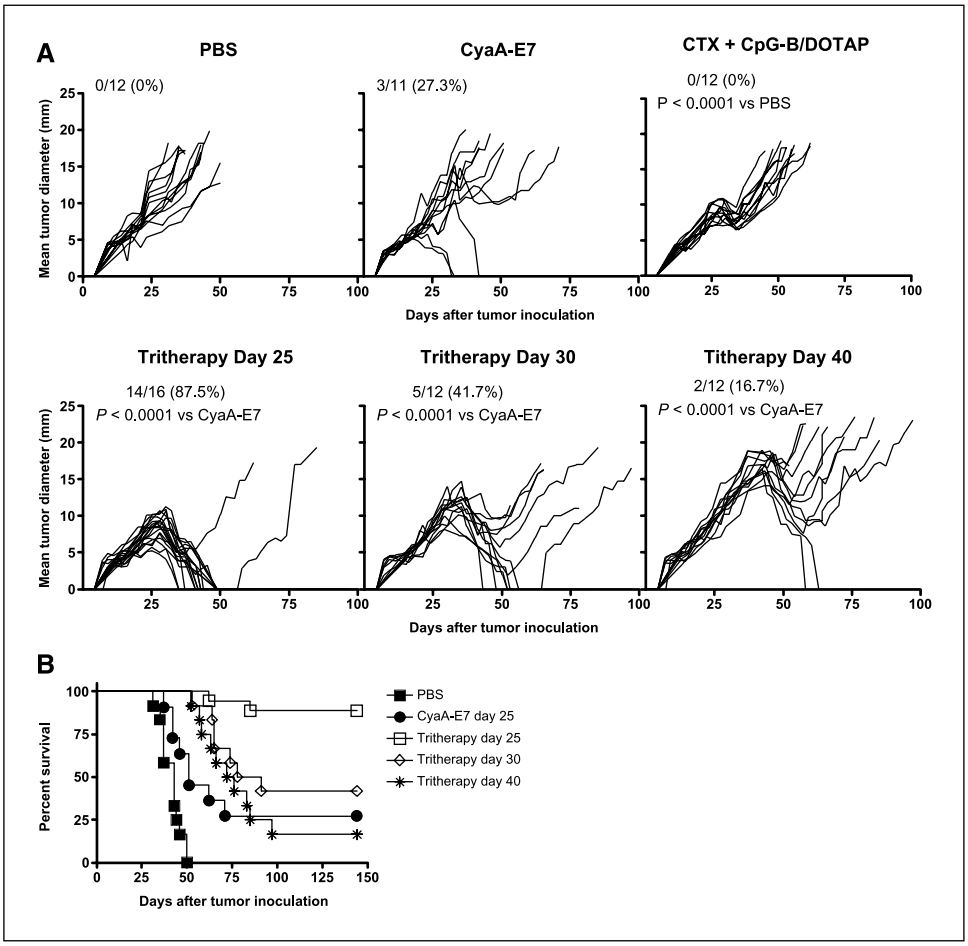

Figure 3. Combined tumor treatment with tritherapy including CyaA-E7, CpG-B/DOTAP, and cyclophosphamide. *A,* C57BL/6 mice were injected on day 0 with 5×10^5 TC-1 cells. On day 24, 29, or 39, they were left untreated or received 2.5 mg cyclophosphamide i.p. On day 25, control mice were injected with PBS or 50 μg CyaA-E7 alone. On day 25, 30, or 40, mice were treated i.v with 50 μg CyaA-E7 and 30 μg CpG-B/DOTAP. The number of tumor-free mice on day 150 relative to the total number of animals included, the percent surviving on day 150, and the *P* value determined by likelihood ratio test comparing tumor growth in various treated groups with tumor growth in the PBS-treated group or CyaA-E7–treated group are indicated for each set of experiments. *B,* Kaplan-Meier plot of mouse survival. The log-rank test was used to compare the groups receiving tritherapy on day 25, 30, or 40 with the group treated with CyaA-E7 alone ($P < 0.0001$). Mice were killed when tumor diameter reached 20 mm or when necessary due to the sanitary status of the animals. Pooled data of two independent experiments.

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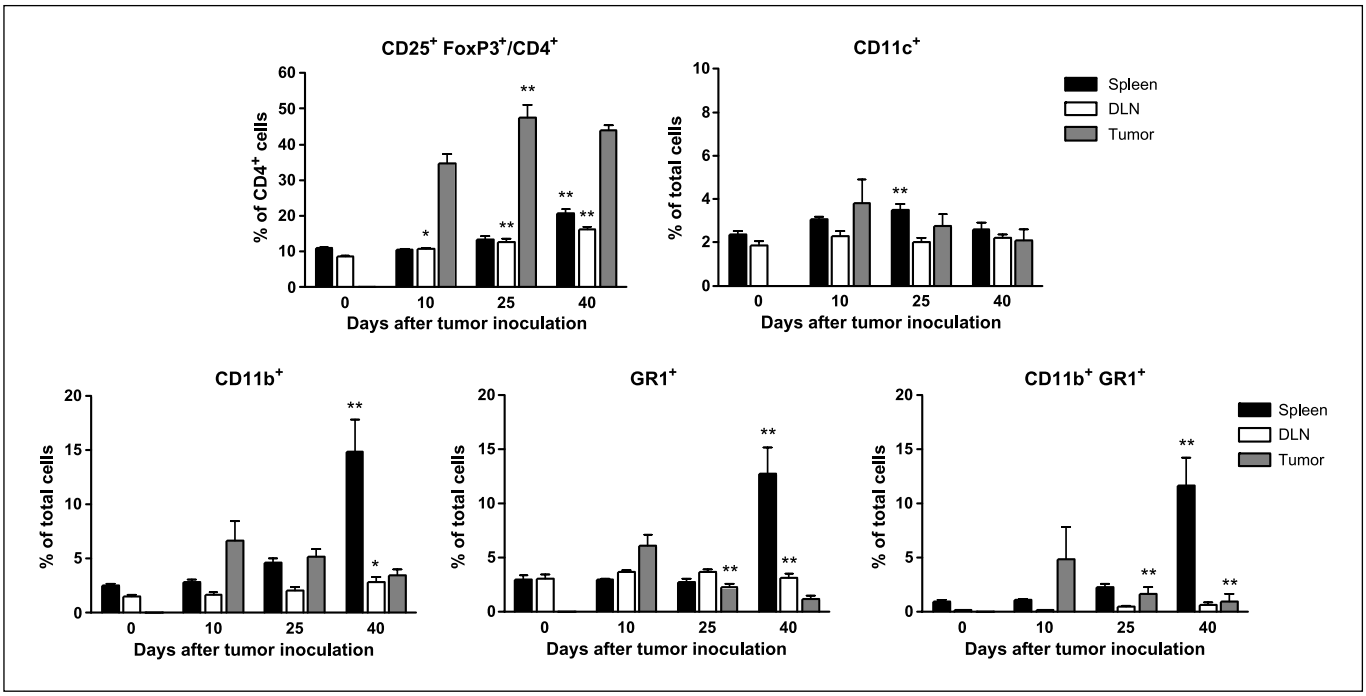

Figure 4. Dynamics of immune cells after tumor cell injection. C57BL/6 mice were injected on day 0 with 5×10^5 TC-1 cells and three mice were killed on days 0, 10, 25, and 40. Spleens, tumor draining lymph nodes (*DLN*), and tumors were excised, ground to prepare the cell suspensions, stained, and analyzed by flow cytometry. Pooled data from two experiments (n = 6 mice). *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. Represented are the percentage of $CD4^+$ cells expressing CD25 and FoxP3, the percentage of $CD11c^+$ cells, the percentage of $CD11b^+$ cells, the percentage of $GR1^+$ cells, and the percentage of $CD11b^+GR1^+$ positive cells.

the tumors, where the percentage of tetramer$^+$ cells reached 70% of total $CD8^+$ cells (Fig. 6). We also observed transient activation of NK cells in spleen and draining lymph node (data not shown), further indicating the onset of the immune response. Anti-CD3 immunohistochemistry revealed a low T-cell infiltration in non-treated tumors that increased dramatically 11 days after tritherapy. Tumors treated on day 40 presented a reduced T-cell infiltration compared with tumor treated on day 25 (Supplementary Fig. S6).

We found a much higher percentage of $CD11b^+GR1^+$ cells (up to 30% of the cells) in the spleen of mice receiving tritherapy than in control mice. Although not as pronounced, there was also a higher percentage of $CD11b^+GR1^+$ cells in the draining lymph node in mice receiving tritherapy than in controls (Fig. 6). This population expressed the specific granulocyte marker Ly6G but not the interleukin 4 receptor α chain. These markers identified this population as nonsuppressive granulocytes/neutrophils (Supplementary Fig. S5B).

Discussion

The recently developed CyaA-E7 vaccine that targets the HPV16-E7 antigen to dendritic cells induces potent antitumor immunity in the absence of adjuvant (11). However, this study shows that the effectiveness of the vaccine progressively diminishes as the time between tumor graft and vaccine administration lengthens; that is, as tumor size at the time of vaccination increases. This experimental situation may reflect what occurs naturally in patients with advanced tumors.

We identified two putative regulatory mechanisms associated with TC-1 tumor growth that may impair the immune response in mice with large TC-1 tumors. First, a high percentage of tumor-infiltrated lymphocytes were $CD25^+FoxP3^+$ Treg (up to 40% of total $CD4^+$ cells). The percentage of $CD25^+FoxP3^+$ cells also increased in draining lymph nodes and spleens as tumors progressed, but remained below 20% of total $CD4^+$ cells. Second, we detected $CD11b^+GR1^+$ myeloid-derived cells in the spleen. Forty days after tumor cell injection, >10% of total spleen cells belonged to this potentially suppressive population. Our findings show that, in this tumor setting, tritherapy has a strong antitumor effect that stimulates adaptive and innate immune responses and simultaneously down-regulates the regulatory components of the immune system.

TLR ligands can be used to activate the innate immune system. Nevertheless, these ligands have various levels of effectiveness and we detected differences even in ligands for the same TLR, for example, TLR-7 ligands R848 and polyuridine complexed with DOTAP. This may be explained by differences in pharmacokinetic profiles of the small-molecule R848 and a liposomal formulation of an oligonucleotide, highlighting a key issue in the development of new adjuvants based on TLR.

We obtained the best results with phosphorothioate CpG oligonucleotides complexed with DOTAP and administered with CyaA-E7: If this combination was administered to mice 25 days after TC-1 injection, the tumors were eradicated in 50% to 60% of treated animals. In the remaining animals, we consistently observed transient arrest of tumor growth followed by relapse. Administration of TLR ligands alone did not affect tumor growth in this large-tumor model. Thus, simultaneous activation of innate (by TLR ligands) and adaptive (by CyaA-E7) components of the immune system were necessary for an efficient therapeutic effect.

We also explored a complementary approach based on the association between immunotherapy and chemotherapy to improve the efficacy of the vaccine. We chose two widely used drugs,

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cyclophosphamide and doxorubicin, that have been shown to enhance antitumor vaccine efficacy (20). I.t. injections of doxorubicin have been reported recently to induce immunogenic cell death, leading to tumor rejection (21). However, in the model used in our study, only cyclophosphamide had adjuvant activity if combined with CyaA-E7, inducing complete tumor regression in ~60% of treated mice. Doxorubicin had no effect when coadministered with CyaA-E7, by the i.t. or i.v. routes (data not shown). A single injection of low-dose cyclophosphamide alone transiently controlled tumor growth, but the tumors started to expand again after a few days in 100% of the animals. As in the case of the TLR ligands, the coadministration of CyaA-E7 with cyclophosphamide was required for such a therapeutic effect. It has been known for decades that low-dose cyclophosphamide exhibits immunopotentiating properties by a mechanism involving the inhibition of a suppressor function (23). More recently, it has been shown that low-dose cyclophosphamide not only decreases the number of Treg but also inactivates the remaining Treg (14, 24–26). Four days after the administration of cyclophosphamide, there were slightly smaller percentages of $CD25^+FoxP3^+$ cells in spleens and draining lymph nodes of these treated mice than in control mice. However, we observed the greatest difference in percentage of these cells in tumor-infiltrated lymphocytes: The percentages of $CD25^+FoxP3^+/CD4^+$ in controls were ~50% and in treated mice, below 30%. This suggests that tumor-associated Treg are more susceptible to cyclophosphamide treatment than are other Treg. Alteration of tumor-associated Treg homeostasis may be a key step in the disturbance of tumor stroma cell interactions after cyclophosphamide treatment (27). Cyclophosphamide treatment was indeed reported to modify the functional profile of tumor-infiltrating T cells, as shown by their inability to produce IFN-γ and to change interleukin 10–producing tumor-infiltrating macrophages into IFN-γ producers. This disturbance, although not sufficient to induce complete regression in our model, may

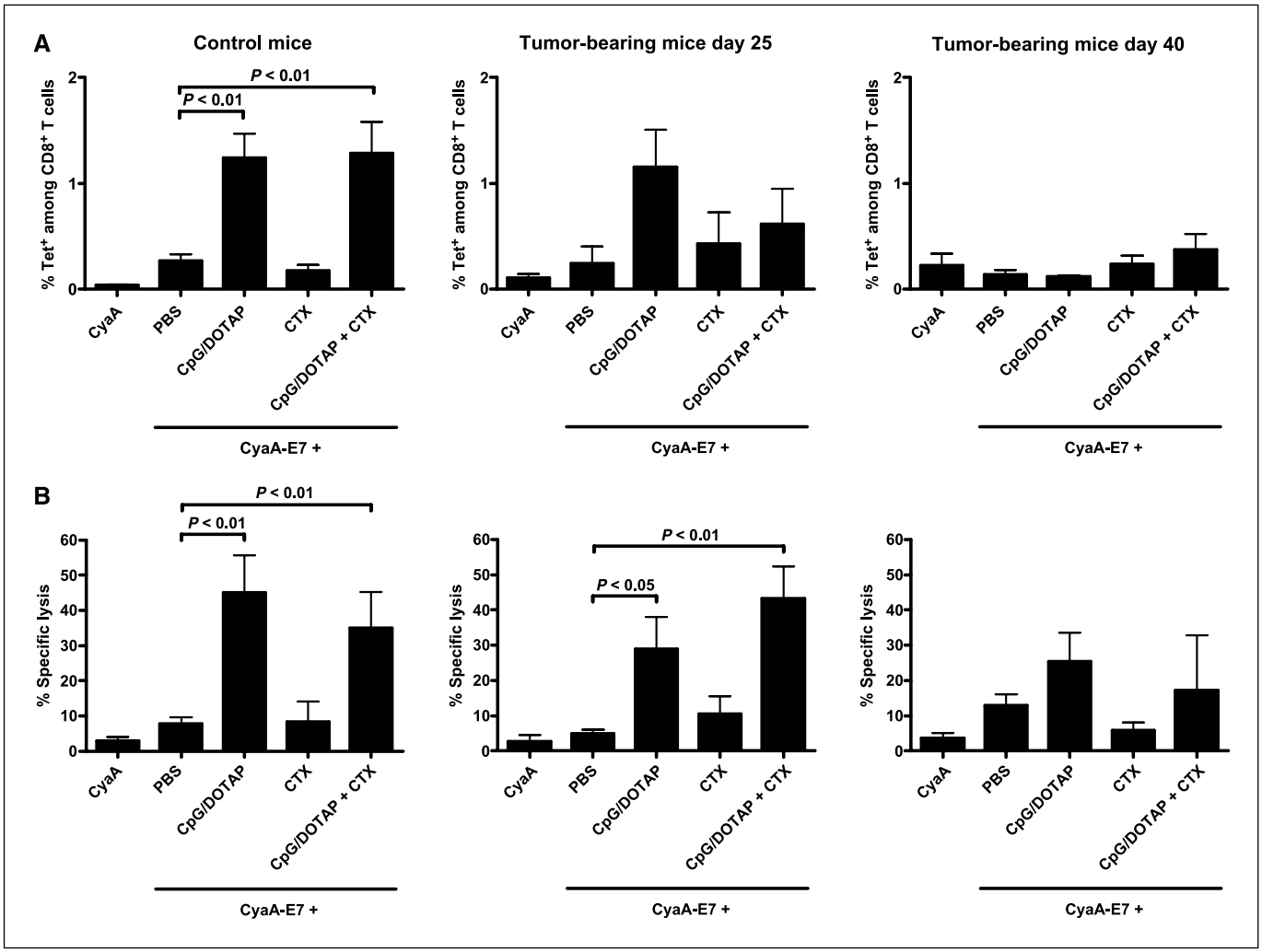

Figure 5. Analysis of tumor-specific immune response in control and tumor-bearing mice after various treatments. C57BL/6 mice were injected on day 0 with 5×10^5 TC-1 cells. Twenty-five or 40 d later, mice were injected with 50 μg control CyaA or CyaA-E7, with PBS or 30 μg CpG-B, and with 2.5 mg cyclophosphamide (24 h before) or both cyclophosphamide and CpG-B/DOTAP. Control mice without tumors received similar treatments. *A,* percentage of $CD8^+$ $tetramer^+$ cells in control mice, in tumor-bearing mice on day 25, and in tumor-bearing mice on day 40. Seven days after treatment, mice were killed and percentages of $tetramer^+$ cells were analyzed by flow cytometry. *B, in vivo* CTL activity in control mice, in tumor-bearing mice on day 25, and in tumor-bearing mice on day 40. Seven days after treatment, mice were injected i.v. with 5×10^6 splenocytes loaded with $CFSE^{high}$ and $E7_{49-57}$ peptide and with 5×10^6 splenocytes loaded with $CFSE^{low}$. Twenty-four hours later, spleens were removed and single-cell suspensions were analyzed by flow cytometry to determine the ratio of $CFSE^{high}$ to $CFSE^{low}$ cells. Pooled data from two experiments (n = 6). *Columns,* mean; *bars,* SE. Data were compared by ANOVA followed by Dunnett's posttest.

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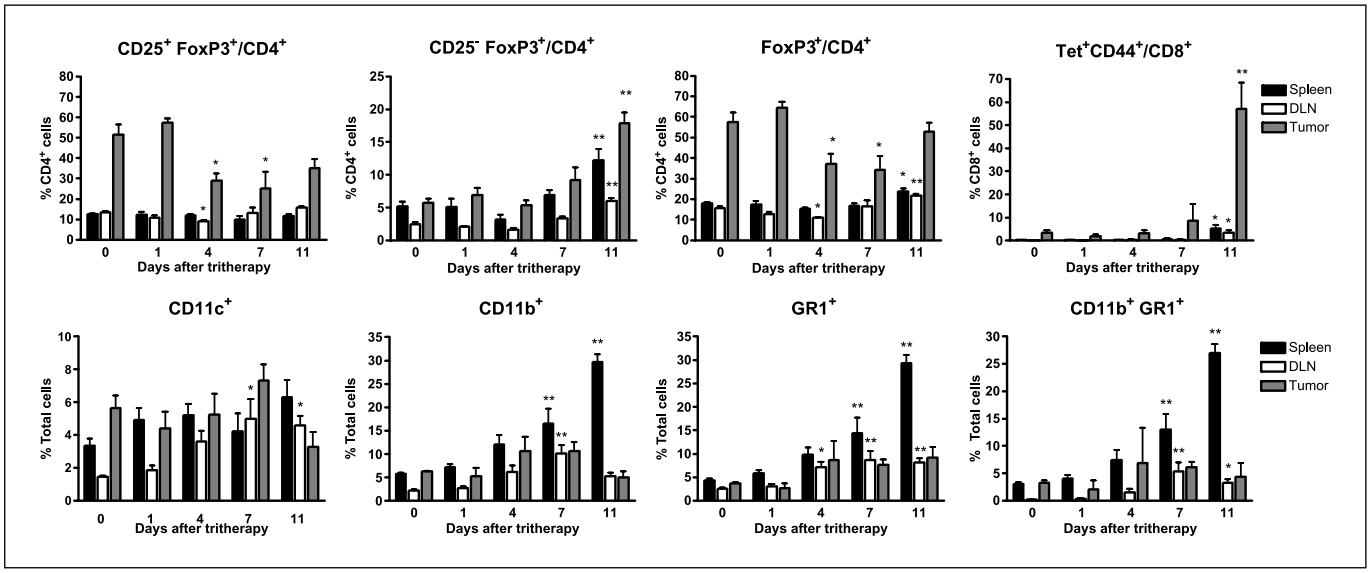

Figure 6. Dynamics of immune cells after tritherapy on day 25. C57BL/6 mice were injected on day 0 with 5×10^5 TC-1 cells. On day 24, they received 2.5 mg cyclophosphamide i.p and 24 h later, 50 μg CyaA-E7 and 30 μg CpG-B/DOTAP. Groups of mice were killed before treatment (day 0); 24 h after cyclophosphamide treatment alone (day 1); or on days 4, 7, or 11 after tritherapy administration. Spleens, tumor draining lymph nodes, and tumors were excised; ground to prepare the cell suspensions; stained; and analyzed by flow cytometry. Pooled data from two experiments (n = 4–6 mice). *Columns,* mean; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$. Represented are the percentage of $CD4^+$ cells that are $CD25^+$ and $FoxP3^+$, the percentage of $CD4^+$ cells that are $CD25^-$ and $FoxP3^+$, the percentage of $CD4^+$ cells that are $FoxP3^+$, the percentage of $CD8^+$ cells that are tetramer$^+$ and $CD44^+$, the percentage of $CD11c^+$ cells, the percentage of $CD11b^+$ cells, the percentage of $GR1^+$ cells, and the percentage of $CD11b^+GR1^+$ cells.

nevertheless pave the way for the action of the effector cells induced by CyaA-E7. Therefore, treatments that enhance effector responses induced by CyaA-E7 should synergize with low-dose cyclophosphamide. Indeed, the combination of low-dose cyclophosphamide with CpG-B/DOTAP and CyaA-E7 had the maximal therapeutic effect in this study. The combination of CpG, cyclophosphamide, and a vaccine in the form of dendritic cell–derived exosomes have been tested previously by Taieb et al. (24) for the treatment of bulky B16A2/gp100 tumors. In this model, however, CpG did not enhance antitumor efficacy of dendritic cell–derived exosomes given in combination with cyclophosphamide. In contrast to these results, we observed strong synergy between the three components, CyaA-E7, CpG-B/DOTAP, and cyclophosphamide, of tritherapy. This tritherapy eradicated large, established tumors in 87% of animals treated 25 days after tumor cell injection, with tumors of a mean diameter of 8 mm. Efficacy was impaired in larger tumors. However, even in mice treated 40 days after tumor cell injection when the mean tumor diameter was higher than 1.5 cm, all tumors transiently shrunk, reflecting the induction of an effective immune response. Tumors were eradicated in 2 of 12 mice with these very large tumors. Moreover, a second administration of the tritherapy 15 days after the first treatment lead to tumor eradication in 43% of treated mice. These results were corroborated with another tumor model based on the injection of transfected EL4 cells that produce the E7 protein. The requirement of the simultaneous targeting of the innate, adaptive, and regulatory components of the immune system has been recently described for the prevention of spontaneous mammary neoplasms (28). In line with our result, total prevention of spontaneous neoplasms required depletion of $CD25^+$ cells, followed by vaccination with a peptide and CpG.

Analyses of immune responses induced by tritherapy showed that the E7-specific $CD8^+$ T-cell response was significantly stronger after administration of the combined CyaA-E7 and CpG/DOTAP treatment, in the absence or presence of cyclophosphamide, than after the administration of CyaA-E7 alone. In tumor-bearing animals, the increase in tetramer$^+$ cells after treatment progressively diminished and these cells were not detected in mice treated 40 days after tumor cell injection. The *in vivo* CTL activity had a similar pattern as that observed for the tetramer analysis. In control mice, the combination of CpG/DOTAP, with or without cyclophosphamide, with CyaA-E7 substantially enhanced E7-specific cytolytic activity. However, this effect was tempered in mice vaccinated 40 days after tumor cell injection: CTL responses were not significantly different from those in control animals. Although tritherapy was more effective against tumors than vaccination with CpG/DOTAP + CyaA-E7, there was no difference between the systemic anti-E7 immune responses induced with or without cyclophosphamide. This supports the hypothesis that the main action of cyclophosphamide in this model is the disturbance of tumor stroma cell interaction.

Treg expansion after vaccination of tumor-bearing hosts has been reported in mice (29, 30) and in humans (31), suggesting that therapeutic cancer vaccines enhance tumor-specific T-cell tolerance. In the tumor model used in this study, whereas the strong antitumor response induced by tritherapy eliminated tumors in ~90% of treated mice, $CD4^+FoxP3^+$ cell expansion occurred in spleens and draining lymph nodes. In addition, the composition of the $CD4^+FoxP3^+$ compartment in spleen, draining lymph node, and tumor was altered after tritherapy, with a larger $CD25^-$ cell population. This may reflect the expansion of tumor-induced regulatory $CD25^-CD4^+$ cells, as reported previously (30). It has been shown that, on a cell per cell basis, $CD4^+CD25^-FoxP3^+$ cells are as regulatory as $CD4^+CD25^+FoxP3^+$ cells (32). We observed substantial expansion of neutrophils in the spleen and draining lymph node after tritherapy. As key component of the inflammatory

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response, neutrophils make important contributions to the recruitment, activation, and programming of antigen-presenting cells and contribute to the quality of the secondary immune response (33, 34). However, neutrophil expansion may detrimentally affect the ongoing antitumor response by the secretion of reactive oxygen species (35).

Finally, our findings show that cyclophosphamide controls the regulatory component of the immune system only during the priming phase of the vaccine. Expansion of regulatory systems during the effector phase induced by tritherapy may abrogate the effector immune response observed in mice immunized 40 days after tumor cell injection. Thus, there is a need for new treatment strategies that favor effector functions by blocking induction or promoting inactivation of the regulatory component of the immune system during the effector phase of the immune response.

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Eradication of Large Tumors in Mice by a Tritherapy Targeting the Innate, Adaptive, and Regulatory Components of the Immune System

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