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Pretreatment with Cisplatin Enhances E7-Specific CD8+ T-Cell–Mediated Antitumor Immunity Induced by DNA Vaccination

Chih-Wen Tseng[1,6], **Chien-Fu Hung**[1,4], **Ronald D. Alvarez**[5], **Cornelia Trimble**[1,2,4], **Warner K. Huh**[5], **Daejin Kim**[1,7], **Chi-Mu Chuang**[1], **Cheng-Tao Lin**[1,6], **Ya-Chea Tsai**[1], **Liangmei He**[1], **Archana Monie**[1], and **T-C. Wu**[1,2,3,4]

[1]Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland [2]Department of Obstetrics and Gynecology, Johns Hopkins Medical Institutions, Baltimore, Maryland [3]Department of Molecular Microbiology and Immunology, Johns Hopkins Medical Institutions, Baltimore, Maryland [4]Department of Oncology, Johns Hopkins Medical Institutions, Baltimore, Maryland [5]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama [6]Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital, Kaohsiung Medical Center, Chang Gung University College of Medicine, Taipei, Taiwan [7]Department of Anatomy, Chung-Ang University College of Medicine, Dongjak-Gu, Seoul, Korea

Abstract

Purpose—Because the combination of multiple modalities for cancer treatment is more likely to generate more potent therapeutic effects for the control of cancer, we have explored the combination of chemotherapy using cisplatin, which is routinely used in chemotherapy for advanced cervical cancer, with immunotherapy using DNA vaccines encoding calreticulin (CRT) linked to human papillomavirus type 16 E7 antigen (CRT/E7) in a preclinical model.

Experimental Design—We characterized the combination of cisplatin with CRT/E7 DNA vaccine using different regimen for its potential ability to generate E7-specific CD8+ T-cell immune responses as well as antitumor effects against E7-expressing tumors.

Results—Our results indicate that treatment of tumor-bearing mice with chemoimmunotherapy combining cisplatin followed by CRT/E7 DNA generated the highest E7-specific CD8+ T-cell immune response and produced the greatest antitumor effects and long-term survival as well as significant levels of E7-specific tumor-infiltrating lymphocytes compared with all the other treatment regimens. Furthermore, we found that treatment with cisplatin leads to the cell-mediated lysis of E7-expressing tumor cells *in vitro* and increased number of E7-specific CD8+ T-cell precursors in tumor-bearing mice. In addition, we observed that E7-specific CD8+ T-cells migrate to and proliferate in the location of TC-1 tumors in mice treated with cisplatin.

Conclusions—Thus, our data suggest that chemoimmunotherapy using cisplatin followed by CRT/E7 DNA vaccine is an effective treatment against E7-expressing tumors and may potentially be translated into the clinical arena.

Requests for reprints: T-C. Wu, Department of Pathology, The Johns Hopkins University School of Medicine, CRB II Room 309, 1550 Orleans Street, Baltimore, MD 21231. Phone: 410-614-3899; Fax: 443-287-4295; wutc@jhmi.edu.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Multimodality treatments that combine conventional cancer therapies with antigen-specific immunotherapy have emerged as promising approaches for the control of cancer (see refs. 1,2 for reviews). Antigen-specific immunotherapy is an attractive approach for the treatment of cancers because it has the potency to specifically eradicate systemic tumors and control metastases without damaging normal cells. A favorable approach to antigen-specific immunotherapy is the use of DNA vaccines based on their safety, stability, and ease of preparation (see refs. 3,4 for review). However, DNA vaccines are poorly immunogenic. Thus, the potency of DNA vaccines needs to be enhanced by using methods to target DNA to the professional antigen-presenting cells and by modifying the properties of antigen-expressing antigen-presenting cells to boost vaccine-elicited immune responses. Several approaches have been developed to enhance DNA vaccine potency (see refs. 5,6 for review).

One particular approach to enhance DNA vaccine potency involves the use of intracellular targeting strategies to enhance MHC class I and class II antigen presentation in dendritic cells. Our previous studies have explored the linkage of calreticulin (CRT), a Ca^{2+}-binding protein located in the endoplasmic reticulum (see ref. 7 for review) to a model tumor antigen, human papillomavirus type 16 (HPV-16) E7, for the development of a DNA vaccine, CRT/E7 (8). We have previously shown that mice vaccinated i.d. with CRT/E7 DNA exhibited a significant increase in E7-specific CD8^{+} T-cell immune response and an impressive antitumor effect against E7-expressing tumors (8). This vaccine was also found to be one of the most effective of the HPV-16 E7 DNA vaccines using intracellular targeting strategies tested (9). These studies suggest that CRT is a highly potent candidate molecule to be used in DNA vaccines targeting HPV infections and HPV-associated lesions.

Antigen-specific DNA vaccines are effective in preclinical models against small tumors. However, such immunotherapeutic strategies alone may not be capable of controlling bulky rapidly growing tumors. This challenge may be overcome by the use of multimodality treatment regimens that combine immunotherapy with chemotherapy to generate a much stronger antitumor effect (10,11). Chemotherapeutic reagents are generally used to treat cancer based on their inherent tendency to attack cells that rapidly proliferate and have a good blood supply. Furthermore, chemotherapeutic reagents travel in the blood system, which allows them to be used for cancers in multiple parts in the body. Cisplatin is one such chemotherapeutic agent that is commonly used to treat certain types of cancers, including ovarian, breast, and cervical cancers (see ref. 12 for review).

In the current study, we have used a combination strategy using CRT/E7 DNA vaccine and cisplatin to generate an enhanced immune response and antitumor effect against E7-expressing tumors. We found that treatment of E7-expressing tumor-bearing mice with cisplatin followed by CRT/E7 DNA generated the strongest E7-specific CD8^{+} T-cell immune responses in the peripheral blood as well as in the tumor and produced the greatest antitumor effects as well as long-term survival compared with all the other treatment regimens. We also found that treatment with cisplatin renders the TC-1 tumor cells more susceptible to lysis by E7-specific CTLs. In addition, we observed that E7-specific CD8^{+} T cells migrate to and proliferate in the location of TC-1 tumors in mice treated with cisplatin. Thus, our data suggest that the chemotherapy using cisplatin followed by immunotherapy using CRT/E7 DNA may represent a potentially feasible approach for the control of HPV-associated malignancies. The clinical implications of this treatment are discussed.

Materials and Methods

Mice

Female C57BL/6 mice (5–8 wk old) were purchased from the National Cancer Institute (Frederick, MD) and kept in the oncology animal facility of the Johns Hopkins Hospital

(Baltimore, MD). All of the animal procedures were done according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Cell line

Briefly, TC-1 cells were obtained by cotransformation of primary C57BL/6 mouse lung epithelial cells with HPV-16 E6 and E7 and an activated *ras* oncogene as described previously (13). The expression of E7 in TC-1 cells has also been characterized previously by He et al. (14).

DNA constructs

The generation of the DNA vaccine encoding CRT and E7(detox) was described previously (9). Briefly, pNGVL4a-CRT/E7(detox) was generated by PCR amplification of CRT by primers (5′-AAAGTCGACATGCTGCTATCCGTGCCGCTGC-3′ and 5′-GAATTCGTTGTCTGGCCGCACAATCA-3′) using a human CRT plasmid as a template. The PCR product was cut with *Sal I/Eco* RI and cloned into the *Sal I/Eco* RI sites of pNGVL4a-E7(detox). The accuracy of DNA constructs was confirmed by DNA sequencing.

DNA vaccination by gene gun

DNA-coated gold particles were prepared, and gene gun particle-mediated DNA vaccination was done according to a protocol described previously (15). Gold particles coated with DNA vaccines (1 µg DNA/bullet) were delivered to the shaved abdominal regions of mice by using a helium-driven gene gun (Bio-Rad Laboratories, Inc.) with a discharge pressure of 400 lb/in^2. C57BL/6 mice (five per group) were immunized with 2 µg of the DNA vaccine and received two boosters with the same dose at 4-d intervals. Splenocytes were harvested 30 d after tumor challenge.

Cisplatin treatment

C57BL/6 mice (five per group) were i.p. injected with cisplatin at a dose of 10 mg/kg body weight twice with a 3-d interval. The given doses were diluted with PBS solution to the required concentration and injected in volumes of 200 µL.

In vivo tumor treatment experiment

For *in vivo* tumor treatment, 1×10^5 TC-1 tumor cells per mouse were injected into 5- to 8-wk-old C57BL/6 mice (five per group) s.c. in the right flank area. After 8 d, the mice were divided into five groups reflecting different treatment regimens: group 1 (five per group) received only TC-1 tumor challenge, group 2 (five per group) was injected with cisplatin as described above, group 3 (five per group) was immunized with the DNA vaccine as described above, group 4 (five per group) was immunized and then injected with cisplatin 4 d later as described above, and group 5 (five per group) was injected with cisplatin and then immunized with the DNA vaccine 4 d later as described above. Mice were monitored twice weekly by inspection and palpation.

Intracellular cytokine staining and flow cytometry analysis

Pooled splenocytes from tumor-bearing and naive mice that were treated with the various treatment regimens were harvested 7 d after the last treatment and incubated for 20 h with 1 µg/mL of E7 peptide containing an MHC class I epitope (amino acids 49–57, RAHYNIVTF) in the presence of GolgiPlug (BD PharMingen; ref. 16). The stimulated splenocytes were then washed once with FACScan buffer and stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8a (clone 53.6.7). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer′s instruction (BD PharMingen). Intracellular IFN-γ was stained with FITC-conjugated rat

anti-mouse IFN-γ. All antibodies were purchased from BD PharMingen. Flow cytometry analysis was done using FACSCalibur with CellQuest software (BD Biosciences).

In vitro CTL assays after cisplatin treatment

Luciferase-expressing TC-1 cells (17) in medium were seeded into a 24-well round-bottomed plate (5×10^4 per well). After sitting overnight, the medium was replaced with 1 mL of fresh medium containing 5 µg cisplatin. The mixture of TC-1 tumor cells and cisplatin-containing medium was incubated in 5% CO_2 for 24 h at 37°C. E7-specific CTLs from the spleens of tumor-bearing mice immunized with the DNA vaccine served as effector cells and were added in the amount of 1×10^6 cells per well. TC-1 cells expressing luciferase were used as target cells. After incubation, D-luciferin (potassium salt; Xenogen Corp.) was added to each well at 150 µg/mL in medium 7 to 8 min before imaging with the Xenogen IVIS 200 system.

In vivo trafficking of E7-specific CD8⁺ T cells expressing luciferase in TC-1 bearing mice

For T-cell imaging, on the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed with 1× HBSS, and finally resuspended with 1× HBSS for injection. Each mouse was challenged with TC-1 (5×10^5) cells s.c. in right flank area. Tumor growth was monitored by visual inspection and palpation for 1 wk. At days 8 and 11, tumor-bearing mice received cisplatin (10 mg/kg) or PBS (control) by i.p. administration. After the last cisplatin injection, tumor-bearing mice received i.v. transfer of luciferase-expressing E7-specific CD8⁺ T cells (E7T-LUC cells; 5×10^6 per mouse; ref. 18). The intensity of bioluminescence was checked before transfer of T cells (i.e., day 0 and on days 3 and 7 after transfer of E7T-LUC cells). Image on day 0 was taken from 3 to 4 h later after transfer of E7T-LUC cells. D-Luciferin was dissolved to 7.8 mg/mL in PBS, filter sterilized, and stored at −80°C. Mice were given D-luciferin by i.p. injection (200 µL/mouse, 75 mg/kg) and anesthetized with isoflurane. *In vivo* bioluminescence imaging for E7T-LUC was conducted on a cryogenically cooled IVIS system (Xenogen) using Living Image acquisition and analysis software (Xenogen). Mice were then placed onto the warmed stage inside the light-tight camera box with continuous exposure to 1% to 2% isoflurane. Images were acquired 7 to 8 min after D-luciferin administration and imaged for 5 min. Generally, three to four mice were imaged at a time. The levels of light from the bioluminescent cells were detected by IVIS camera system, integrated, and digitized. Region of interest from displayed images was designated around the tumor sites and quantified as total photon counts using Living Image 2.50 software (Xenogen).

Characterization of CD11b⁺ Gr1⁺ myeloid suppressor cells

C57BL/6 mice (five per group) were inoculated with TC-1 tumor cells at a dose of 9×10^5. Mice were treated with cisplatin at a dose of 10 mg/kg body weight on days 9 and 12 after tumor challenge. Blood samples and splenocytes were harvested on day 26 after tumor challenge and characterized for CD11b⁺Gr1⁺ myeloid suppressor cells using antibodies specific for CD11b and Gr1 (BD PharMingen). The staining was characterized by flow cytometry analysis. Flow cytometry analysis was done using FACSCalibur with CellQuest software.

Statistical analysis

Data presented as mean ± SE are representative of at least two different experiments. All *P* values <0.05 were considered significant. Statistical analysis was done using independent sample *t* test. Survival and the event time distributions for different mice were compared by Kaplan-Meier analysis and by use of the log-rank statistics. Graphs were done using SigmaPlot program file (Systat Software, Inc.).

Results

TC-1 tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA generate the best therapeutic antitumor effects

To determine the antitumor effects of chemoimmunotherapy combining cisplatin and DNA-encoding CRT linked to the mutated form of E7 [CRT/E7(detox)], we first challenged groups of C57BL/6 mice (five per group) with TC-1 tumor cells and then treated them with the different regimens of cisplatin and/or the DNA vaccine as illustrated in Fig. 1A. As shown in Fig. 1B, tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA showed significantly lower tumor volumes over time compared with tumor-bearing mice treated with the other treatment regimens ($P < 0.005$). Furthermore, tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA showed improved survival compared with tumor-bearing mice treated with the other treatment regimens ($P < 0.05$; Fig. 1C). Thus, our data suggest that the treatment regimen using cisplatin followed by CRT/E7(detox) DNA produces the best therapeutic antitumor effects and long-term survival in TC-1 tumor-bearing mice.

TC-1 tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA generate the highest frequency of E7-specific CD8^{+} T cells

To determine the E7-specific CD8^{+} T-cell immune response in mice treated with the various regimens, we first challenged groups of C57BL/6 mice (five per group) with TC-1 tumor cells and then treated them with DNA vaccine alone, DNA vaccine followed by cisplatin, or cisplatin followed by DNA vaccine as illustrated in Fig. 1A. As a control, a group of naive C57BL/6 mice was also treated with similar regimens for comparison. Seven days after the last treatment, we harvested splenocytes from vaccinated mice and characterized them for the presence of E7-specific CD8^{+} T cells using intracellular cytokine staining for IFN-γ followed by flow cytometry analysis. As shown in Fig. 2, tumor-bearing mice that were given cisplatin followed by CRT/E7(detox) DNA generated a significantly higher number of E7-specific CD8^{+} T cells compared with tumor-bearing mice that were given CRT/E7(detox) DNA followed by cisplatin or DNA alone ($P < 0.005$). Similarly, we also observed higher numbers of E7-specific CD8^{+} T cells in naive mice treated with cisplatin followed by CRT/E7(detox) DNA compared with naive mice treated with CRT/E7(detox) DNA followed by cisplatin or DNA alone ($P < 0.005$). However, the enhancement of the E7-specific CD8^{+} T cells generated by treatment with cisplatin followed by CRT/E7(detox) DNA was more pronounced in tumor-bearing mice compared with naive mice. Thus, our results suggest that treatment of tumor-bearing mice with cisplatin followed by CRT/E7(detox) DNA leads to the strongest E7-specific CD8^{+} T-cell immune response.

Treatment of tumor-bearing mice with cisplatin leads to increased number of E7-specific CD8^{+} T-cell precursors

To determine the effect of treatment of HPV-16 E7-expressing tumor-bearing mice with cisplatin, we first challenged C57BL/6 mice (five per group) with TC-1 tumors and treated them with or without cisplatin. Twenty days after the cisplatin treatment, splenocytes were harvested and characterized for the presence of E7-specific CD8^{+} T cells using intracellular cytokine staining from IFN-γ followed by flow cytometry analysis. As shown in Fig. 3, TC-1 tumor-bearing mice treated with cisplatin showed significantly increased numbers of E7-specific CD8^{+} T-cell precursors compared with tumor-bearing mice without cisplatin treatment ($P < 0.005$). We also characterized the E7-specific CD4^{+} T-cell immune responses in cisplatin-treated mice. However, no significant CD4^{+} T-cell immune responses were observed in the treated mice (data not shown). Thus, our data suggest that chemotherapy with cisplatin leads to an increase in the E7-specific CD8^{+} T-cell response.

Treatment with cisplatin increases MHC class I expression and renders the TC-1 tumor cells more susceptible to lysis by E7-specific CTLs

Previous studies have shown that the expression of MHC class I was enhanced after cisplatin treatment, suggesting that cisplatin induces tumor-specific antitumor immunity (19,20). We further characterized the expression of MHC class I in E7-expressing TC-1 tumors following treatment with cisplatin. We found that treatment with cisplatin increased the MHC class I expression in TC-1 tumors (see Supplementary Fig. S1). To determine if treatment of TC-1 tumor cells with cisplatin will render the tumor cell more susceptible to E7-specific T-cell–mediated killing, we did a cytotoxicity assay using luciferase-expressing TC-1 tumor cells. TC-1 tumor cells were treated with 5 µg/mL cisplatin alone, treated with 5 µg/mL cisplatin and 1×10^6 E7-specific CTLs, or treated with 1×10^6 E7-specific CTL alone. Untreated TC-1 tumor cells were used as a control. The CTL-mediated killing of the TC-1 tumor cells in each well was monitored using bioluminescent imaging systems. The degree of CTL-mediated killing of the tumor cells was indicated by the decrease of luminescence activity. As shown in Fig. 4, the lowest luciferase activity was observed in the wells incubated with cisplatin and E7-specific CTLs compared with the wells incubated with cisplatin alone or E7-specific CTL alone ($P < 0.005$). Thus, our data suggest that the TC-1 tumor cells treated with cisplatin show increased MHC class I expression, leading to increased susceptibility of the tumor cells for lysis by the E7-specific CTLs.

E7-specific CD8^{+} T cells migrate to and proliferate in the location of TC-1 tumors in mice treated with cisplatin

To determine whether the luciferase-expressing E7-specific CD8^{+} T cells can migrate to and proliferate in the location of TC-1 tumor cells in tumor-bearing mice treated with cisplatin, we did adoptive T-cell transfer experiments using E7T-LUC cells in mice with established TC-1 tumors. C57BL/6 mice (five per group) were s.c. challenged with 5×10^5 TC-1 tumor cells per mouse. On days 8 and 11, the mice were treated i.p. with either cisplatin or PBS (control). One week after the last cisplatin injection, TC-1 tumor-bearing mice were i.v. given 5×10^6 cells per mouse of E7-specific CD8^{+} T cells expressing luciferase (E7T-LUC). The location as well as the number of E7T-LUC cells were characterized using bioluminescent imaging systems. As shown in Fig. 5A, there was a significantly higher number of E7T-LUC cells at the site of TC-1 tumor cells in tumor-bearing mice treated with cisplatin on day 7 compared with the untreated tumor-bearing mice. The luminescence activity of the E7T-LUC cells in the tumor-bearing and naive mice was quantified in the form of line graphs (Fig. 5B). There was a significant increase in the luminescence activity in the tumor-bearing mice treated with cisplatin over time compared with untreated tumor-bearing mice. Thus, our data suggest that E7T-LUC cells migrate to and proliferate in the location of TC-1 tumors in tumor-bearing mice treated with cisplatin.

TC-1 tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA generated significant levels of E7-specific tumor-infiltrating lymphocytes of vaccinated mice

To determine if treatment with cisplatin followed by CRT/E7(detox) DNA vaccination in TC-1 tumor-bearing mice will lead to an increase in the number of E7-specific CD8^{+} T-cell immune responses in the tumor-infiltrating lymphocytes (TIL), we did intracellular cytokine staining followed by flow cytometry analysis. We first challenged groups of C57BL/6 mice (five per group) with TC-1 tumor cells and then treated them with the different regimens as illustrated in Fig. 1A. As shown in Fig. 6, we observed a significantly higher number of E7-specific CD8^{+} T cells in TILs of the TC-1 tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA compared with those in tumor-bearing mice treated with the other treatment regimens. Thus, our data indicate that TC-1 tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA generated significant levels of E7-specific TILs of vaccinated mice.

Treatment with cisplatin generated significantly lower levels of myeloid suppressor cells and T regulatory cells in the blood and spleen of TC-1 tumor-bearing mice

To determine if treatment with cisplatin will lead to a decrease in the number of myeloid suppressor cells in peripheral blood and spleens of TC-1 tumor-bearing mice, we first challenged groups of C57BL/6 mice (five per group) with TC-1 tumor cells and, 9 days later, treated them with or without cisplatin at the dose of 10 mg/kg body weight twice with a 3-day interval. Twenty-six days after tumor challenge, the cells isolated from blood and spleen were harvested and characterized for $CD11b^+Gr1^+$ myeloid suppressor cells using flow cytometry analysis. As shown in Fig. 7, we observed a significantly lower number of $CD11b^+Gr1^+$ myeloid suppressor cells in the peripheral blood and spleens of tumor-bearing mice treated with cisplatin followed by CRT/E7(detox) DNA compared with tumor-bearing mice treated with the other treatment regimens. We also characterized the number of $CD4^+CD25^+$ T regulatory cells in the blood and spleens of tumor-bearing mice following cisplatin treatment. We observed that tumor-bearing mice showed significantly increased levels of $CD4^+CD25^+$ T regulatory cells compared with mice without tumor. Furthermore, we found that cisplatin treatment led to the reduction of $CD4^+CD25^+$ T regulatory cells in the peripheral blood mononuclear cells and splenocytes of tumor-bearing mice (Supplementary Fig. S2). Thus, our data indicate that treatment with cisplatin significantly reduces the levels of myeloid suppressor cells and T regulatory cells in tumor-bearing mice.

Discussion

In the current study, we tested the efficacy of combination therapy using cisplatin and CRT/E7 DNA vaccine. Our data suggest that treatment with cisplatin renders the TC-1 tumor cells more susceptible to lysis by E7-specific CTLs and leads to increased number of E7-specific $CD8^+$ T-cell precursors in tumor-bearing mice. Furthermore, we found that treatment of tumor-bearing mice with cisplatin followed by CRT/E7 DNA generated the strongest E7-specific $CD8^+$ T-cell immune responses, the greatest antitumor effects and long-term survival, as well as significant levels of E7-specific TILs compared with all the other treatment regimens. Thus, our data suggest that treatment using cisplatin followed by CRT/E7 DNA vaccine may represent an effective therapeutic regimen for the control of HPV-associated malignancies.

Our results are consistent with a previous observation that combinational therapy using chemotherapy with immunotherapy generated a better therapeutic effect compared with chemotherapy or immunotherapy alone. Previously, Bae et al. (21) have shown that cisplatin combined with HPV E7 peptide-based subunit vaccines improved the cure of established E7-expressing tumors as well as the long-term antitumor immunity in tumor-bearing mice. Their data also suggest that treatment with cisplatin increased the sensitivity of the tumors to CTL-mediated killing, which agrees with our current data (see Fig. 4). Taken together, these data indicate that chemotherapy with cisplatin can be used in combination with different forms of antigen-specific immunotherapy.

Our results have shown that cisplatin followed by CRT/E7 DNA treatment represents the best regimen to generate a strong E7-specific immune response and antitumor effect compared with all the other treatment regimens. However, it is interesting to note that the reverse treatment involving administration of the DNA vaccine before cisplatin administration failed to result in a similar antigen-specific $CD8^+$ T-cell immune response. Several reasons may account for the observed phenomenon. First, cisplatin is known to induce cell death through apoptosis or necrosis (see ref. [22] for review). Thus, treatment of tumor-bearing mice with cisplatin will lead to the delay or inhibition of tumor growth. Second, pretreatment of the tumor with cisplatin leads to the apoptosis of the tumor cells, causing the uptake of E7 antigen by the antigen-presenting cells and activation of E7-

specific CD8$^+$ T cells. Third, treatment with cisplatin also renders E7-expressing tumors more susceptible to killing by E7-specific cytotoxic CD8$^+$ T cells. All these factors contribute to the significant expansion of E7-specific CD8$^+$ T cells as well as TILs, resulting in a significant antitumor effect against E7-expressing tumors.

We also observed that treatment with cisplatin causes a significant reduction in the number of myeloid suppressor cells in tumor-bearing mice. We believe that this reduction in the number of myeloid suppressor cells may be related to a smaller tumor load in tumor-bearing mice receiving treatment with cisplatin compared with untreated mice. It has been reported that the numbers of myeloid suppressor cells closely correlate with the size of the tumor (23). However, we cannot exclude the possibility of the direct influence of the therapeutic regimen on the number of myeloid suppressor cells in treated mice.

In the future, it will be important to explore the effect of other chemotherapeutic agents in combination with various DNA vaccination strategies on the treatment of tumors. Thus, this study shows the effectiveness and clinical feasibility of using immunotherapy as a complement to chemotherapy to enhance the antitumor immunity induced by DNA vaccination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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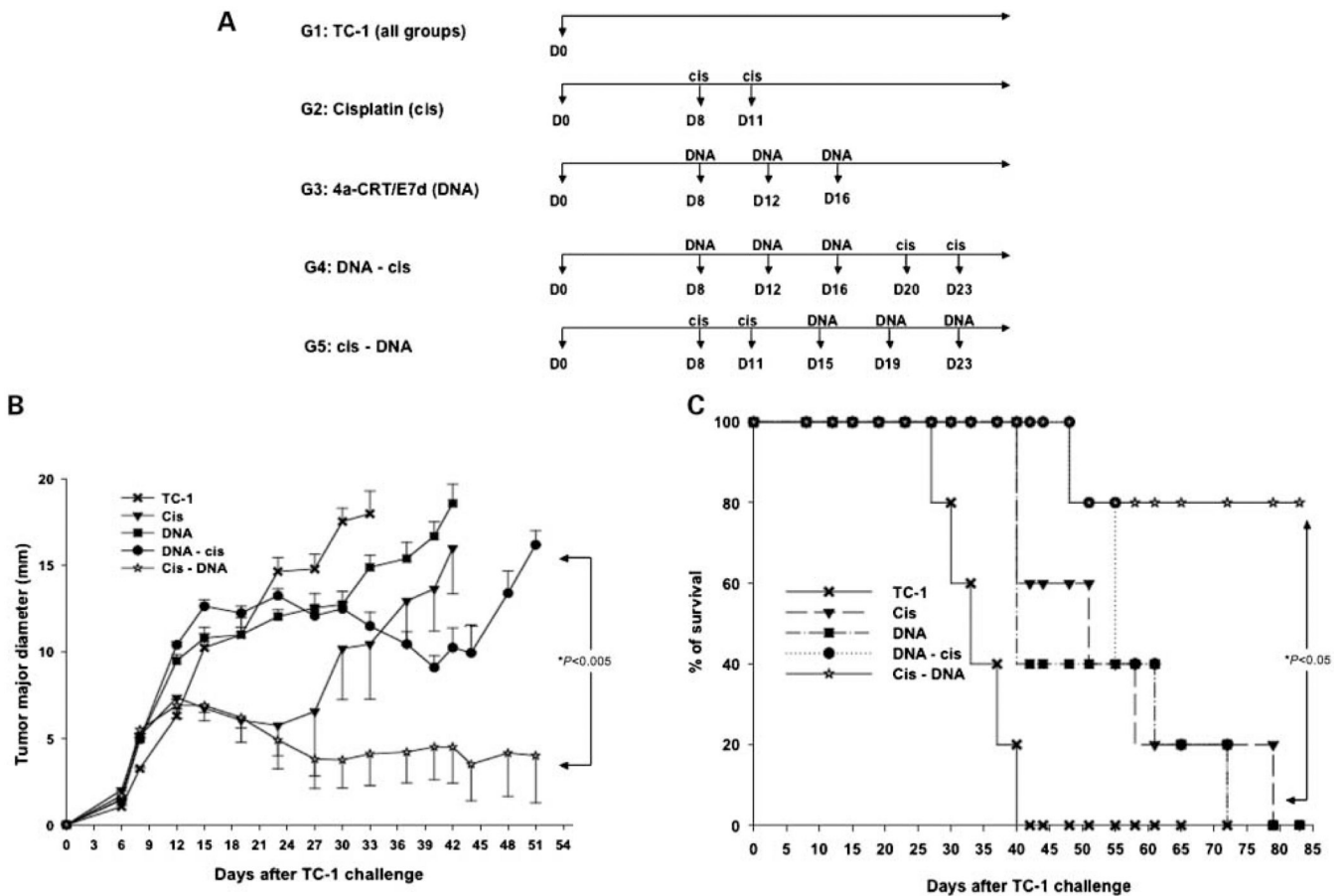
Fig. 1.
In vivo tumor treatment experiments. Groups of C57BL/6 mice (five per group) were s.c. challenged with 1×10^5 per mouse of TC-1 tumor cells on day 0. Tumor-bearing mice were treated with cisplatin (*cis*) and/or DNA-encoding CRT/E7 (*DNA*) as indicated in the time line. Cisplatin was given via i.p. injection of 10 mg/kg body weight. DNA was given via gene gun in the amount of 2 µg/mouse. *A*, diagrammatic representation of the different treatment regimens of cisplatin and/or DNA vaccine. *B*, line graph depicting the tumor volume in TC-1 tumor-bearing mice treated with the different treatment regimens. Points, mean; bars, SE. Note that the group of tumor-bearing mice treated with cisplatin followed by the DNA vaccine had the best therapeutic antitumor effect over time compared with tumor-bearing mice treated with the other treatment regimens ($P < 0.005$). *C*, Kaplan-Meier survival analysis of TC-1 tumor-bearing mice treated with the different treatment regimens. Note that the tumor-bearing mice treated with cisplatin followed by DNA vaccine showed improved survival compared with tumor-bearing mice treated with the other treatment regimens ($P < 0.05$).
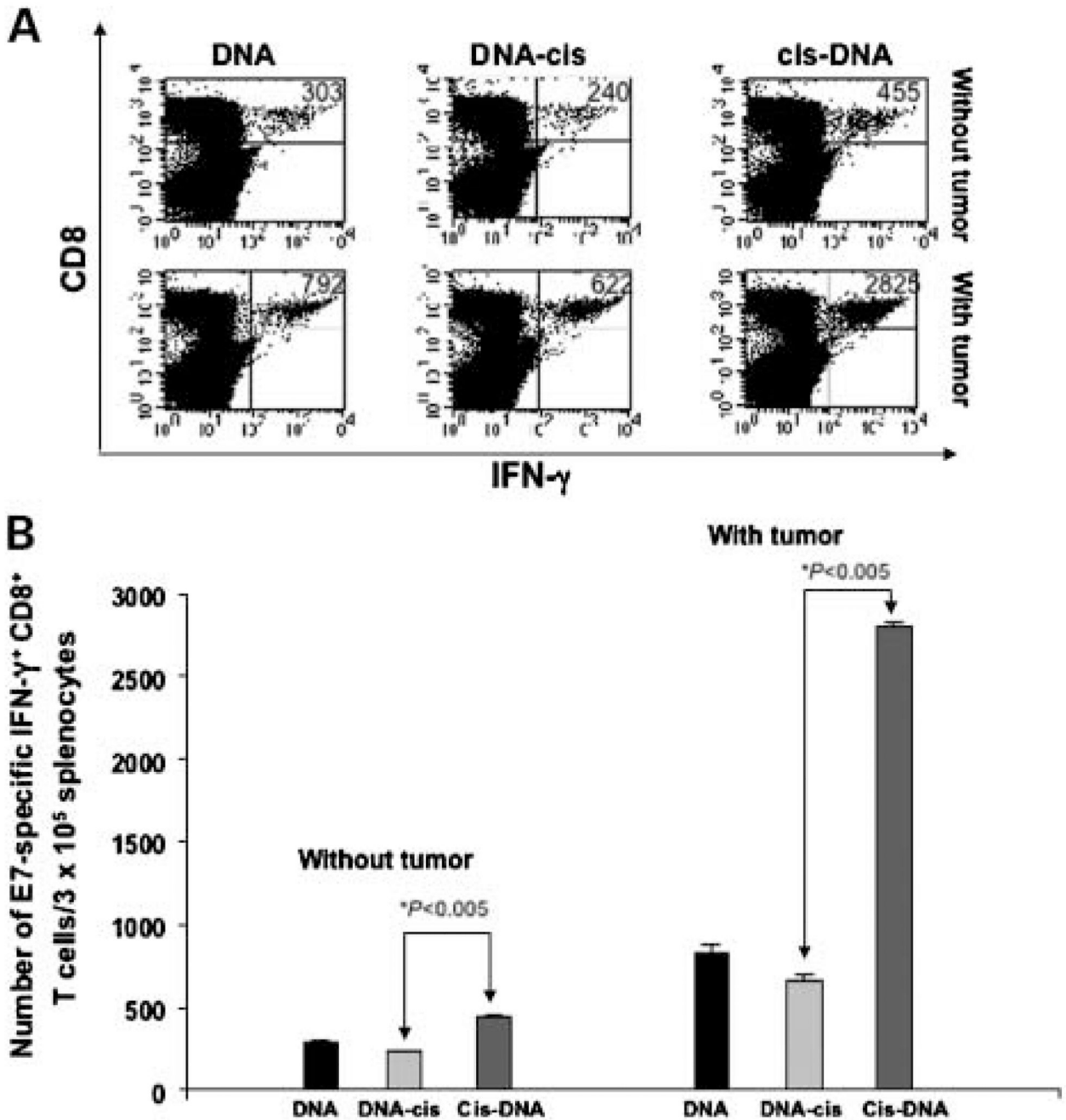
Fig. 2.
Intracellular cytokine staining followed by flow cytometry analysis to determine the number of E7-specific CD8^{+} T cells in tumor-bearing mice treated with cisplatin and/or DNA vaccine. Groups of C57BL/6 mice (five per group) were challenged with TC-1 tumor cells and treated with cisplatin and/or DNA as illustrated in Fig. 1*A*. Naive C57BL/6 mice (five per group) were also given cisplatin and/or DNA following the same regimen as tumor-bearing mice for comparison. Thirty days after tumor challenge, splenocytes from mice with and without tumor challenge were harvested and incubated with E7 peptide overnight. Determination of the E7-specific CD8^{+} T cells was done by intracellular IFN-γ staining followed by flow cytometry analysis. *A*, top right quadrant, representative data of

intracellular cytokine stain followed by flow cytometry analysis showing the number of E7-specific IFN-γ$^+$ CD8$^+$ T cells in the various groups. *B*, bar graph depicting the numbers of E7-specific IFN-γ– producing CD8$^+$ T cells per 3×10^5 pooled splenocytes. Columns, mean; bars, SE.
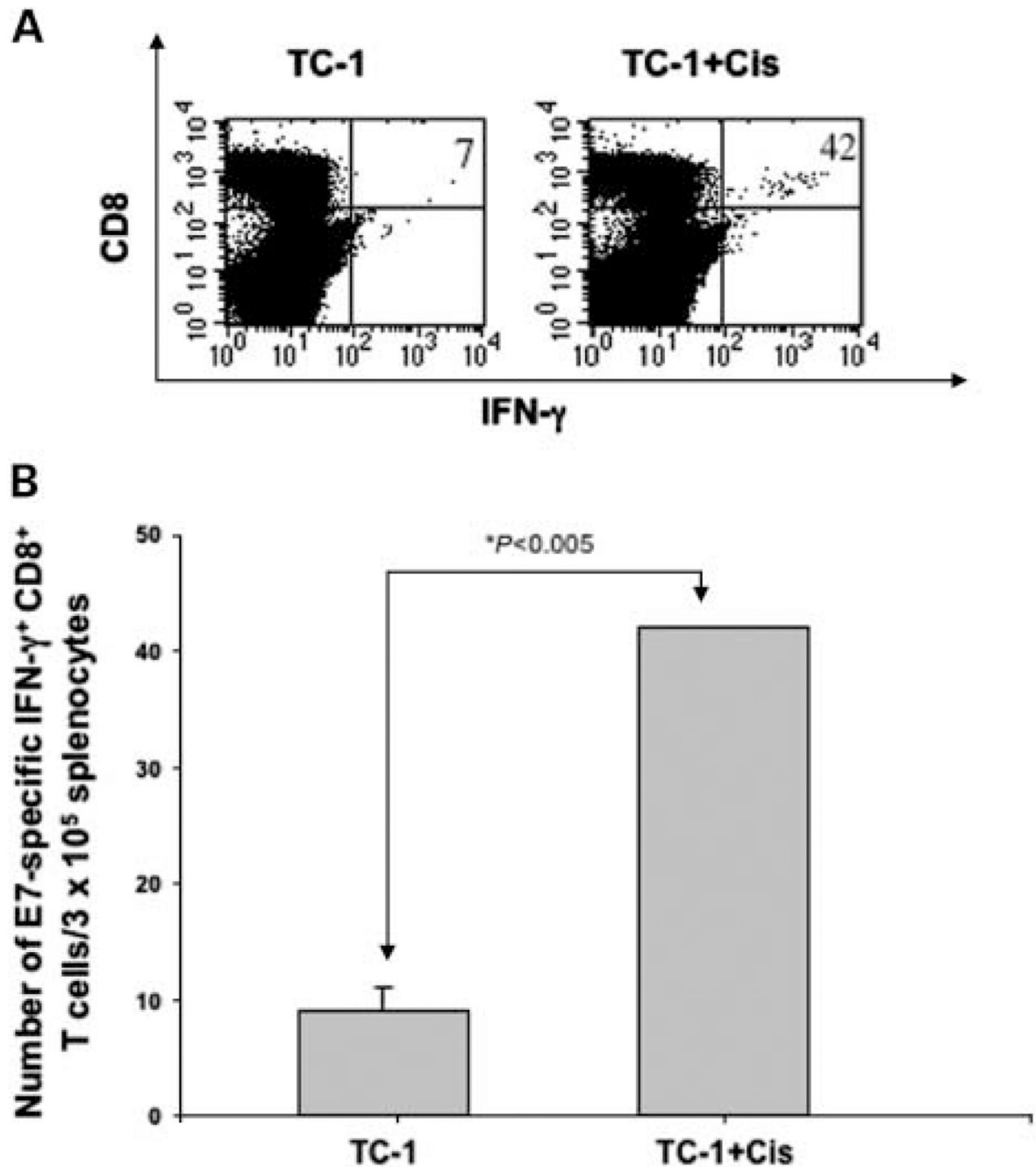
Fig. 3.
Intracellular cytokine staining followed by flow cytometry analysis to determine the number of E7-specific CD8^{+} T cells in tumor-bearing mice treated with or without cisplatin. Groups of C57BL/6 mice (five per group) were challenged with TC-1 tumor cells and treated with or without cisplatin at the dose of 10 mg/kg body weight twice with a 3-d interval. Thirty days after tumor challenge, splenocytes from nontreated and treated mice were harvested and incubated with E7 peptide overnight. Determination of the E7-specific CD8^{+} T cells was done by intracellular IFN-γ staining followed by flow cytometry analysis. *A*, representative data of intracellular cytokine stain followed by flow cytometry analysis showing the number of E7-specific IFN-γ^{+} CD8^{+} T cells in the different groups. *B*, bar

graph depicting the numbers of E7-specific IFN-γ– producing CD8$^+$ T cells per 3×10^5 pooled splenocytes. Columns, mean; bars, SE. Note that the TC-1 tumor-bearing mice treated with cisplatin showed significantly increased levels of E7-specific CD8$^+$ T cells ($P < 0.005$).
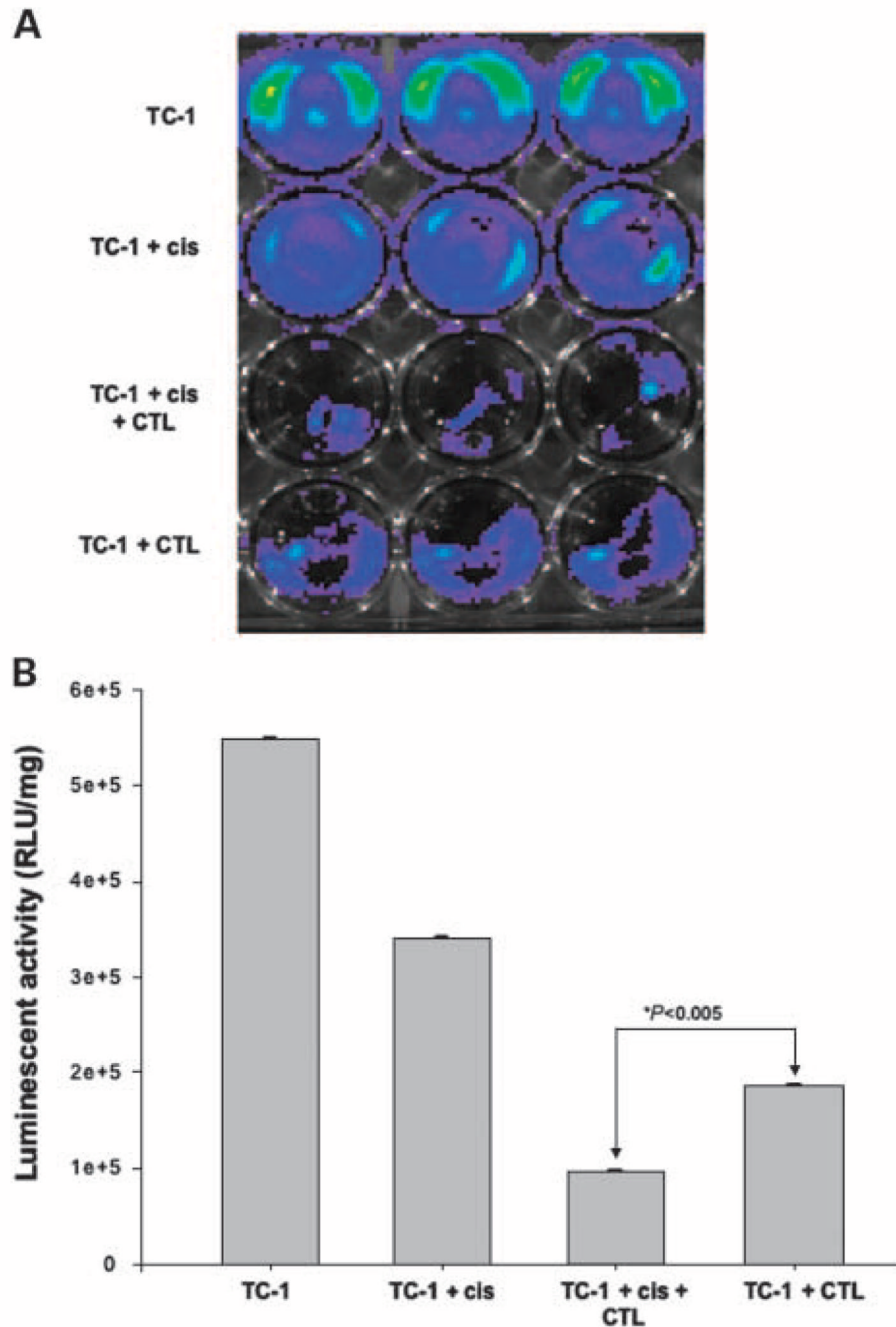
Fig. 4.
In vitro cytotoxicity assay. Luciferase-expressing TC-1 tumor cells were added to 24-well plates at a dose of 5×10^4 per well. Three days later, TC-1 tumor cells were untreated, treated with 5 µg/mL cisplatin alone, treated with 5 µg/mL cisplatin and 1×10^6 E7-specific CTLs, or treated with 1×10^6 E7-specific CTL alone. The degree of CTL-mediated killing of the tumor cells was indicated by the decrease of luminescence activity using the IVIS Luminescence Imaging System Series 200. Bioluminescence signals were acquired for 1 min. *A*, representative luminescence images of 24-well plates showing lysis of the tumor cells. *B*, bar graph depicting the quantification of luminescence intensity in tumor cells treated with cisplatin and/or E7-specific CTLs. Columns, mean; bars, SE. Note that the TC-1

tumor cells treated with cisplatin and E7-specific CTLs led to significant loss of luminescence intensity indicating enhanced lysis of tumor cells by the E7-specific CD8^{+} T cells ($P < 0.005$). RLU, relative luciferase unit.
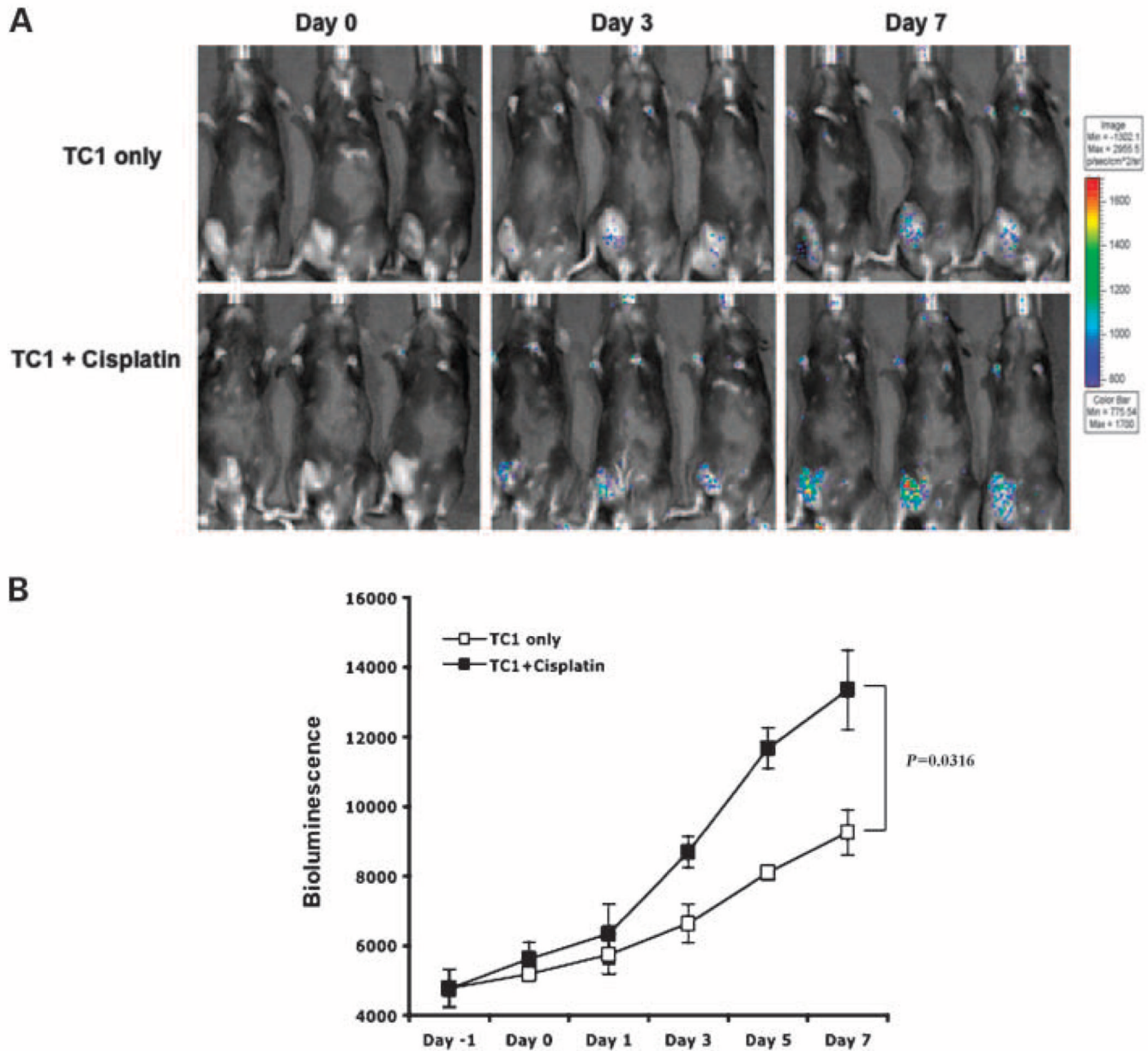
Fig. 5.
Characterization of luciferase-expressing E7-specific CD8^{+} T cells in TC-1 tumor-bearing mice treated with or without cisplatin using luminescence imaging. Groups of C57BL/6 mice (five per group) were challenged with 5×10^5 per mouse of TC-1 tumor cells. On days 8 and 11, the mice were treated either with cisplatin at the dose of 10 mg/kg body weight or with PBS (control) by i.p. administration. One week after the last cisplatin injection, TC-1 tumor-bearing mice were i.v. given 5×10^6 cells per mouse of E7-specific CD8^{+} T cells expressing luciferase (E7T-LUC). Mice were imaged using the IVIS Imaging System Series 200. Bioluminescence signals were acquired for 5 min on days 0, 3, and 7 after adoptive transfer treatment. *A*, representative luminescence images of tumor-bearing mice treated with or without cisplatin on 0, 3, and 7 d after adoptive transfer treatment of E7T-LUC cells. *B*, line graph depicting the kinetic expression of luciferase over a period of 7 d in the tumor-bearing mice treated with or without cisplatin. Points, mean; bars, SE.
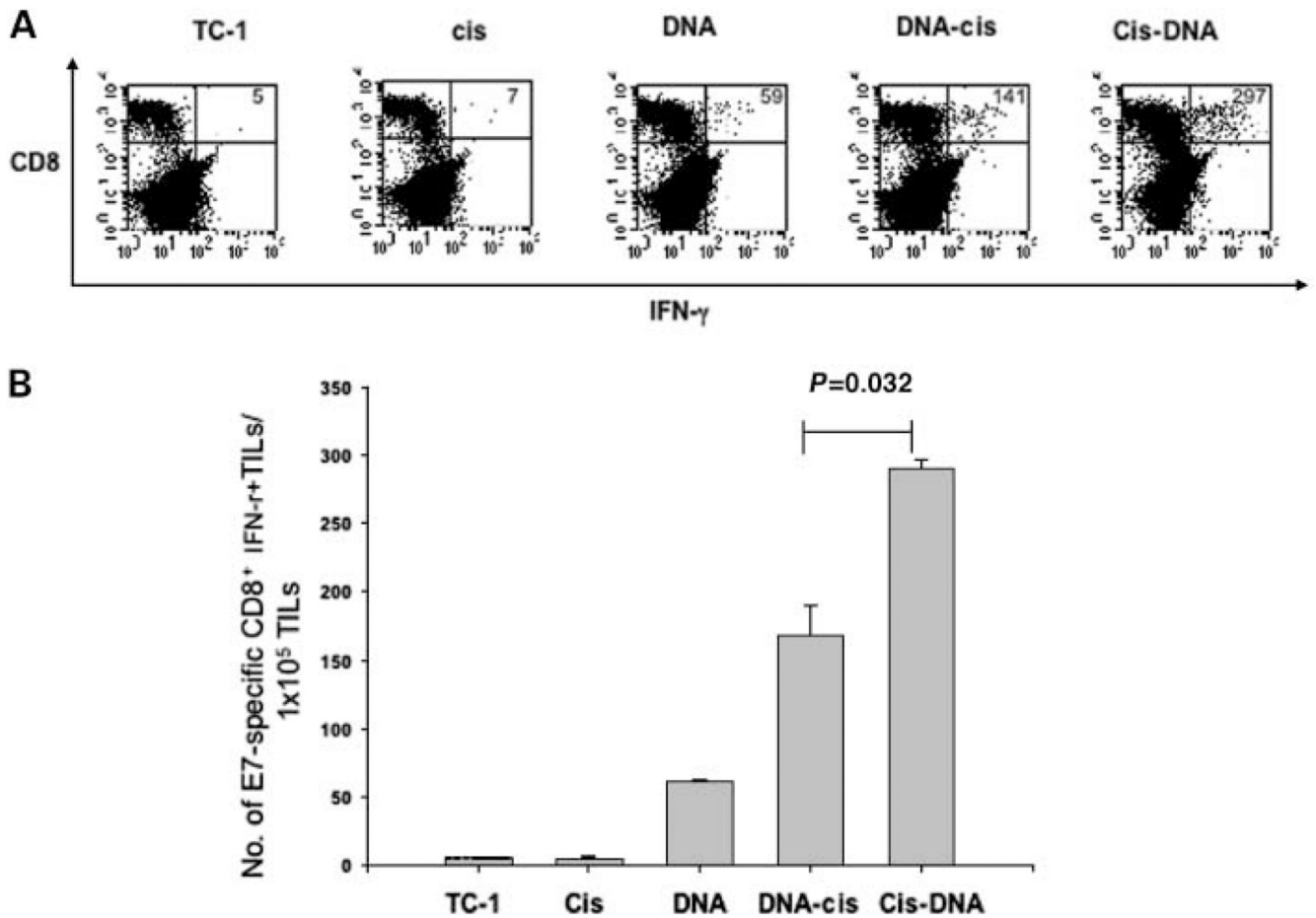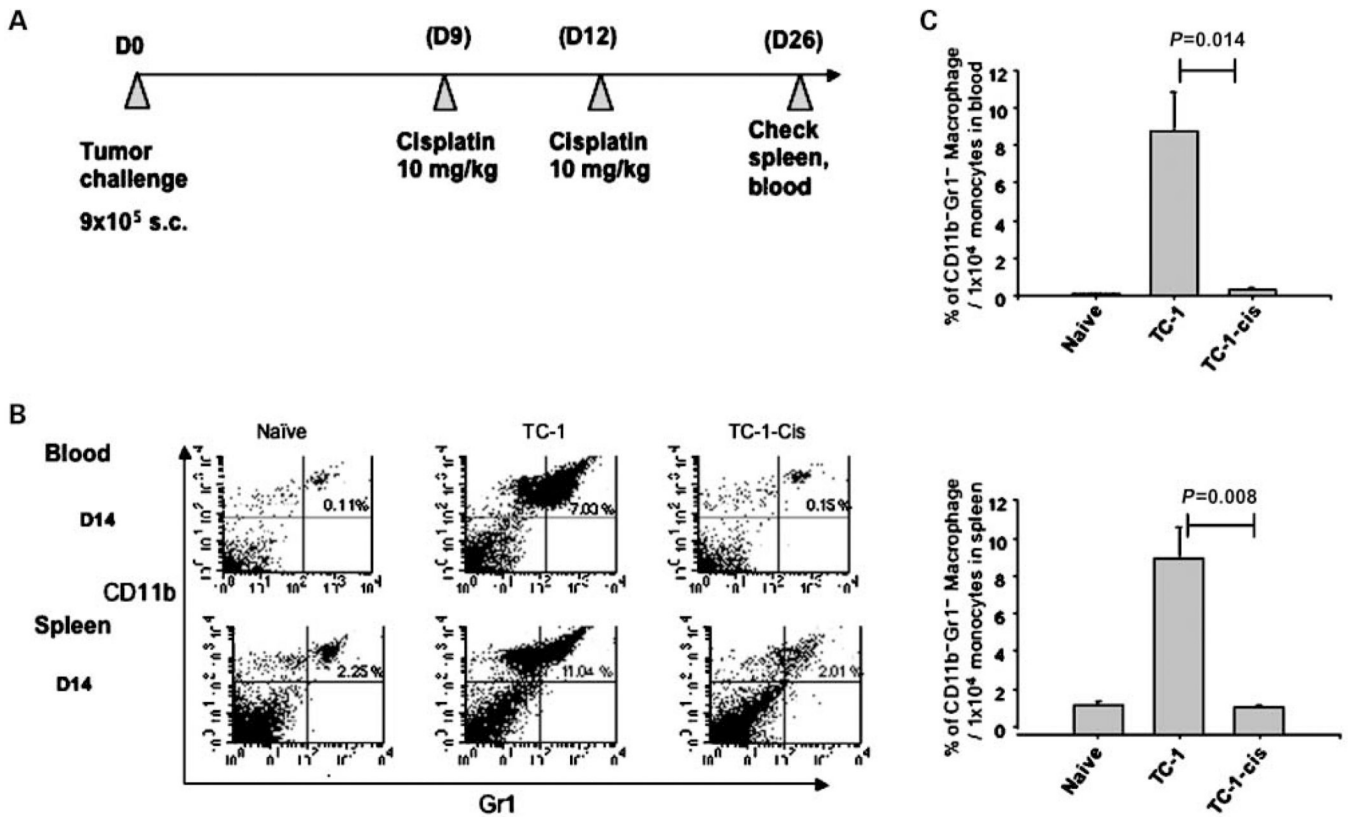
Fig. 6.
Intracellular cytokine staining followed by flow cytometry analysis to determine the number of E7-specific CD8^{+} TILs in tumor-bearing mice treated with cisplatin and/or DNA vaccine. Groups of C57BL/6 mice (five per group) were challenged with TC-1 tumor cells and treated with cisplatin and/or DNA as illustrated in Fig. 1*A*. Thirty days after tumor challenge, TILs from mice were harvested and stained for CD8 and intracellular IFN-γ and then characterized for E7-specific CD8^{+} TILs using intracellular IFN-γ staining followed by flow cytometry analysis. *A*, top right quadrant, representative data of intracellular cytokine staining followed by flow cytometry analysis showing the number of E7-specific IFN-γ^{+} CD8^{+} TILs in the various groups. *B*, bar graph depicting the numbers of E7-specific IFN-γ– producing CD8^{+} TILs per 1×10^5 TILs. Columns, mean; bars, SE.

Fig. 7.
Intracellular cytokine staining followed by flow cytometry analysis to determine the number of CD11b$^+$Gr1$^+$ myeloid suppressor cells in tumor-bearing mice treated with or without cisplatin. Groups of C57BL/6 mice (five per group) were challenged with 9×10^5 per mouse of TC-1 tumor cells. Nine days later, the mice were treated with or without cisplatin at the dose of 10 mg/kg body weight twice with a 3-d interval. Blood samples and splenocytes were harvested on day 26 after tumor challenge. The cells isolated from blood and spleens were then characterized for CD11b$^+$Gr1$^+$ myeloid suppressor cells using flow cytometry analysis. *A*, diagrammatic representation of the treatment regimen of cisplatin. *B*, representative flow cytometry analysis data showing the number of CD11b$^+$Gr1$^+$ myeloid suppressor cells in the different groups. *C*, bar graph depicting the numbers of CD11b$^+$Gr1$^+$ myeloid suppressor cells per 1×10^4 monocytes in the blood or in the spleen. Columns, mean; bars, SE.