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Turning On/Off Tumor-Specific CTL Response during Progressive Tumor Growth[1]

Yujun Huang, Nikolaus Obholzer, Raja Fayad, and Liang Qiao[2]

Therapeutic vaccinations used to induce CTLs and treat firmly established tumors are generally ineffective. To understand the mechanisms underlying the failure of therapeutic vaccinations, we investigated the fate of tumor-specific CD8$^+$ T cells in tumor-bearing mice with or without vaccinations. Our data demonstrate that tumor-specific CD8$^+$ T cells are activated at the early stage of tumor growth, tumor-specific CTL response reaches a maximal level during progressive tumor growth, and tumor-specific CD8$^+$ T cells lose cytolytic function at the late stage of tumor growth. The early stage therapeutic vaccination induces efficient antitumor activity by amplifying the CTL response, whereas the late-stage therapeutic vaccination is invalid due to tumor-induced dysfunction of CD8$^+$ T cells. However, at the late stage, tumor-specific CD8$^+$ T cells are still present in the periphery. These tumor-specific CD8$^+$ T cells lose cytolytic activity, but retain IFN-γ secretion function. In contrast to in vitro cultured tumor cells, in vivo growing tumor cells are more resistant to tumor-specific CTL killing, despite an increase of tumor Ag gene expression. Both tumor-induced CD8$^+$ T cell dysfunction at the late stage and immune evasion developed by in vivo growing tumor cells contribute to an eventual inefficacy of therapeutic vaccinations. Our study suggests that it is important to design a vaccination regimen according to the stages of tumor growth and the functional states of tumor-specific CD8$^+$ T cells. ***The Journal of Immunology,*** **2005, 175: 3110–3116.**

The identification of tumor-associated Ags recognized by cytotoxic CD8$^+$ T lymphocytes (1–3) and eradication of tumors by adoptive transfer of tumor-specific CD8$^+$ T cells (4) demonstrates that CD8$^+$ T cells play an important role in antitumor immunity, which provides the rationale of the development of CTL-based tumor vaccines. Preventive vaccinations or vaccinations early after tumor cell inoculation can be effective in rejection of inoculated tumor cells. However, therapeutic vaccinations used to treat established tumors are generally ineffective (5–7).

The failure of therapeutic vaccinations could be due to tumor-induced tolerance of tumor-specific CD8$^+$ T cells. Tumor-specific CD8$^+$ T cells in tumor-bearing hosts appear to have different fates in different tumor models. In the nonself tumor Ag models, the lymphocytic choriomeningitis virus (LCMV)[3] Ag was used as a tumor Ag, and LCMV-specific CD8$^+$ T cells in the spleen and tumor-infiltrating cells were not tolerized by the tumor expressing the LCMV Ag (8, 9). In a model of endogenous tumors expressing the LCMV Ag as a self tumor Ag, Nguyen et al. (10) found that tumor growth enhanced cross-presentation leading to limited CD8$^+$ T cell activation without tolerance, and these T cells remained functional to subsequent virus challenge. However, tumor-specific CD8$^+$ T cells can be tolerized in tumor-bearing hosts. Shrikant et al. (11, 12) showed that the tumor-expressing OVA tolerized adoptively transferred OVA-specific CD8$^+$ T cells by inhibiting CD8$^+$ T cell proliferation.

In this study, we use human papillomavirus (HPV)-16 E7 as a model Ag to study the mechanisms underlying tumor resistance to therapeutic vaccinations. HPV-16 E7 protein is an oncoprotein that causes human cervical cancer. Because this viral Ag is a foreign Ag for the tumor-bearing host, which excludes the existence of self-tolerance of E7-specific CD8$^+$ T cells, it is an attractive target for cancer immunotherapy. We s.c. inoculated RMA-E7 cells into mice to generate E7$^+$ tumors. E7 DNA vaccine was used to induce E7-specific CTL response. We sought to determine whether the tumor induces activation or tolerance of tumor-specific CD8$^+$ T cells and how therapeutic vaccination influences the CTL response and tumor growth. Using C57BL/6 mice, a more physiologically relevant model without unusual transgenic T cells, we tracked the fate of E7-specific CD8$^+$ T lymphocytes in response to E7$^+$ tumors and/or vaccinations. We found that tumor-specific CD8$^+$ T cells were activated at the early stage of tumor growth, tumor-specific CTL response reached a maximal level during progressive tumor growth, and tumor-specific CD8$^+$ T cells lost cytolytic function at the late stage of tumor growth. The early stage therapeutic vaccination induced efficient antitumor activity by amplifying the CTL response, whereas the late-stage therapeutic vaccination was invalid due to tumor-induced dysfunction of tumor-specific CD8$^+$ T cells. The late-stage, tumor-specific CD8$^+$ T cells lost cytolytic activity, but retained IFN-γ secretion function in response to Ag stimulation. Our data suggest that defining the specific stages of functional states of tumor-specific CD8$^+$ T cells is essential for effective therapeutic vaccinations.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. Mice were maintained under specific pathogen-free conditions in the Loyola University Comparative Medicine Facility. All of the experimental procedures were conducted according to the protocols approved by the Institutional Animal Care and Use Committee.

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; HPV, human papillomavirus; PBST, PBS-0.05% Tween 20.

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Cell lines

RMA-neo cell line is a murine lymphoma cell line RMA (H-2^b) transfected with empty vector plasmid pCI-neo (Promega). RMA-E7 is the RMA cell line transfected with pCI-E7 encoding HPV-16 E7 (13). Both RMA-neo and RMA-E7 cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM glutamine, 5×10^{-5} M 2-ME, and 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 (all were obtained from Invitrogen Life Technologies). Cells were cultured in a humidified incubator at 37°C with 5% v/v CO_2.

Tumor cell inoculations and vaccinations

Six- to 8-wk-old C57BL/6 mice were used for experiments. A total of 5×10^4 RMA-E7 cells was s.c. injected into each mouse to generate E7$^+$ tumors. For vaccinations, 50 μg of plasmid DNA pCMV-E7 encoding a double mutant E7 (13) was s.c. injected into each mouse with 20 μg of bovine papillomavirus virus-like particles as the adjuvant (14) to enhance the CTL response. Tumor inoculations and vaccinations were given at indicated time points. The tumor growth was recorded twice every week.

IFN-γ ELISPOT assay

An IFN-γ ELISPOT assay was used to detect $E7_{49-57}$ RAHYNIVTF epitope-specific IFN-γ secreting cells after in vitro stimulation with synthetic $E7_{49-57}$ peptide. Multiscreen 96-well plates (Millipore) were coated with 100 μl/well of 5 μg/ml anti-IFN-γ capture Ab (BD Pharmingen) in PBS at 4°C overnight. Plates were washed once with 200 μl/well complete RPMI 1640 and blocked with 200 μl/well complete RPMI 1640 at room temperature for 2 h. Splenocytes were added starting at 5×10^5 cells/well in triplicate wells with 1/3 serial dilutions. Cells were cultured in complete RPMI 1640 containing 50 U/ml IL-2 (R&D Systems) and 5 μg/ml H-2 D^b-restricted $E7_{49-57}$ peptide. LCMV-specific D^b-restricted CTL epitope peptide GP_{33-41} was used as the negative control. $E7_{49-57}$ (RAHYNIVTF) and GP_{33-41} (KAVYNFATC) peptides were synthesized by Biosynth International. The purity was >95%. After a 40-h incubation at 37°C and 5% CO_2, plates were washed two times with deionized water and three times with 200 μl/well PBS-0.05% Tween 20 (PBST). Then, plates were incubated for 1 h at room temperature with 100 μl/well of 2.5 μg/ml biotinylated anti-IFN-γ detection Ab (BD Pharmingen) in PBS-10% FBS. After washing three times with PBST, 100 μl/well avidin-HRP (eBioscience) was added to the wells at 1/1000 dilution in PBS-10% FBS, and plates were incubated at room temperature for 1 h. After washing four times with PBST and 2 times with PBS, 100 μl/well of 3-amino-9-ethylcarbazole substrate solution (Sigma-Aldrich) were added to the wells, and the spots were developed at room temperature for 20–60 min until visible spots appeared. The reaction was stopped by washing plates three times with 200 μl/well distilled water. The plates were air-dried, and the spots were counted by using a dissecting microscope.

In vitro cytotoxicity assay

The cytotoxicity was measured by a standard 6-h ^{51}Cr-release assay. Splenocytes were isolated from mice in each group and enriched for T cells by nylon-wool column (Polysciences). T cell-enriched splenocytes were seeded into 96-well U-bottom plates (BD Discovery Labware) at 5×10^5 cells/well in 200 μl of complete RPMI 1640 with 5 μg/ml $E7_{49-57}$ peptide and 5% T-stim without ConA (BD Discovery Labware). A total of 1×10^5 cells/well irradiated (3000 rad) splenocytes from naive mice was used as feeder cells. After in vitro stimulation for 5 days, splenocytes were used as effector cells for the ^{51}Cr-release assay. A total of 1×10^6 in vitro cultured RMA-E7 cells or RMA-E7 tumor cells freshly isolated from in vivo growing tumors were used as target cells by incubating at 37°C with 200 μCi of sodium ^{51}Cr (PerkinElmer) for 1 h. RMA-neo cells, RMA-neo cells pulsed with 5 μg/ml LCMV GP_{33-41} peptide, or RMA-neo cells pulsed with 5 μg/ml $E7_{49-57}$ peptide were also used as target cells to test E7-specific cytolytic activity in all of the ^{51}Cr-release assays. Target cells were seeded into 96-well V-bottom plates (Nunc) at 2×10^3 cells/well in 100 μl of complete RPMI 1640. Effector cells were seeded into triplicate wells containing the target cells at various E:T cell ratios, making a final volume of 200 μl. Plates were incubated at 37°C in a humidified incubator with 5% v/v CO_2 for 6 h. Plates were centrifuged, and 100 μl of supernatant was removed from each well to assess chromium release using a gamma radiation counter (PerkinElmer). The percentage of specific lysis (%) was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Experimental release represents the mean radioactivity released by target cells in the presence of effector cells. Maximal release represents the radioactivity released after total lysis of target cells with 2% Triton X-100. Spontaneous release represents the radioactivity released by target cells present in the medium without effector cells.

Cell lysate preparation, SDS-PAGE, and Western blot analysis

Tumors were excised from tumor-bearing mice 21–30 days after tumor inoculation. Tumor tissues were disrupted by homogenization and passage through a 70-μm nylon cell strainer (BD Discovery Labware). Cells were washed in complete RMPI 1640 and purified for live cells by centrifugation on a Ficoll gradient (Amersham Biosciences). Interphase cells from the gradient were pooled and washed. To prepare the cell lysates for Western blot, RMA-E7 in vitro cultured cells and freshly isolated tumor cells were lysed using 100 μl of lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, and 50 mM Tris-HCl (pH8.0)) per 1×10^7 cells. The soluble cell lysates were collected, and the same amount of total proteins was loaded for each sample. Samples were separated by 15% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell Microscience). Western blotting was performed using mouse mAb anti-HPV 16 E7 (Zymed Laboratores) and HRP-conjugated sheep anti-mouse IgG (Amersham Biosciences). Finally, the membranes were processed with an ECL system (Amersham Biosciences) followed by exposure to the x-ray film (Kodak).

Results

Therapeutic vaccinations slow down tumor growth, but are not sufficient for tumor regression

To investigate the mechanisms underlying the failure of therapeutic vaccinations against tumors, we used HPV-16 E7 as a tumor Ag to establish a tumor model: 5×10^4 RMA-E7 cells were s.c. injected into each C57BL/6 mouse to generate E7$^+$ tumors; and the DNA vaccine pCMV-E7 encoding a mutated E7 was s.c. injected to induce E7-specific CTLs. The tumor inoculation and therapeutic vaccination protocol is shown in Fig. 1*A*. On day 12 after tumor cell inoculation, mice with established tumors were used for therapeutic vaccinations. Two weeks later, mice were boosted once. Tumor-bearing mice without therapeutic vaccinations or mice treated with vaccinations alone were used as the controls. As shown in Fig. 1*B*, therapeutic vaccinations slowed down tumor growth, but were not sufficient for tumor regression. At the late stage, the tumor grew up progressively in the presence of vaccinations. Therefore, we hypothesized that the established late-stage tumor induced tumor-specific CD8$^+$ T cell tolerance, which led to the unresponsiveness following vaccinations.

The established late-stage tumor causes the loss of cytolytic activity of tumor-specific CD8$^+$ T cells during tumor growth

To determine whether tumor-induced tolerance of tumor-specific CD8$^+$ T cells occurs at the late stage of tumor growth, on day 33, 7 days after the second vaccination as shown in Fig. 1*A*, mice were sacrificed, and splenocytes were isolated to test the cytolytic activity of E7-specfic CTLs by in vitro ^{51}Cr-release assays. Splenocytes from each mouse were in vitro stimulated with $E7_{49-57}$ peptide for 5 days and then were used as effector cells. In vitro cultured RMA-E7 cells were used as the target cells for E7-specific CTL killing. Background lysis on RMA-neo cells or RMA-neo cells pulsed with LCMV GP_{33-41} peptide was below 10% at the highest E:T ratio (data not shown). As shown in Fig. 2*A*, there was a high level of E7-specific cytolytic activity in mice treated with vaccinations alone, which indicates that our DNA vaccination protocol can induce functional E7-specific CTLs. However, there was no significant level of E7-specific cytolytic activity in tumor-bearing mice with or without therapeutic vaccinations. Lysis on RMA-neo cells pulsed with $E7_{49-57}$ peptide was similar to lysis on RMA-E7 cells (data not shown). It suggests that the established late-stage tumor causes the loss of cytolytic activity of tumor-specific CD8$^+$ T cells, which might result in the failure of therapeutic vaccinations. However, therapeutic vaccinations did slow down the tumor growth, which suggests that vaccinations have an antitumor effect at the early stage.

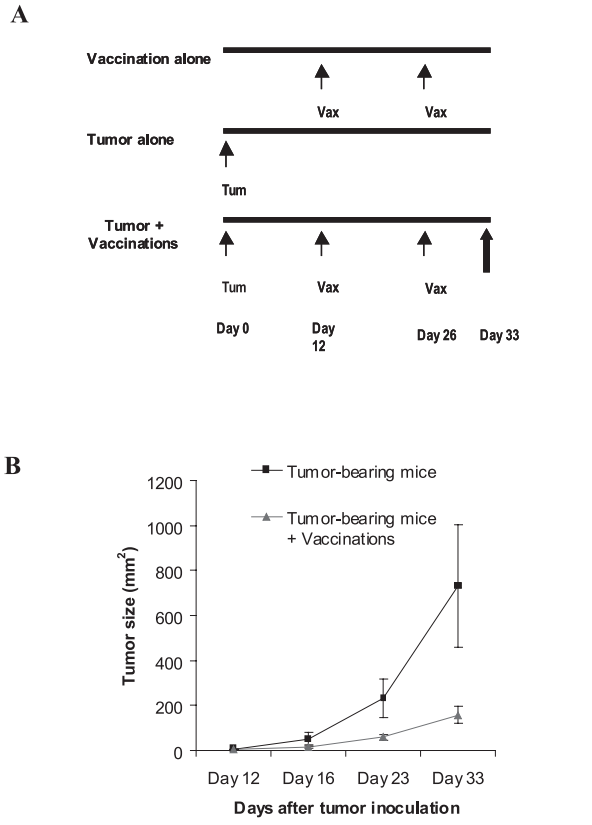

FIGURE 1. Therapeutic vaccinations slow down tumor growth, but are not sufficient for tumor regression. *A*, At day 0, 5×10^4 RMA-E7 cells were s.c. injected into each mouse to generate $E7^+$ tumors. At day 12, tumor growth was checked, and mice with established tumor were defined as tumor-bearing mice. In the tumor plus vaccinations group, tumor-bearing mice were vaccinated with E7 DNA vaccine on day 12 and boosted on day 26. On day 33, mice were sacrificed to test E7-specific CTL response. In the tumor alone group, tumor-bearing mice were not vaccinated. For the vaccination alone group, mice were vaccinated at the same time points as in the tumor plus vaccinations group. *B*, In tumor-bearing mice with or without vaccinations, the growing tumors were measured at indicated time points. The data represented the average sizes for four mice per group. The experiments were performed independently at least five times with the similar results. The difference between two groups was statistically significant. ANOVA followed by Turkey *t* test was used for statistical analysis, and a $p < 0.05$ was accepted as statistically significant.

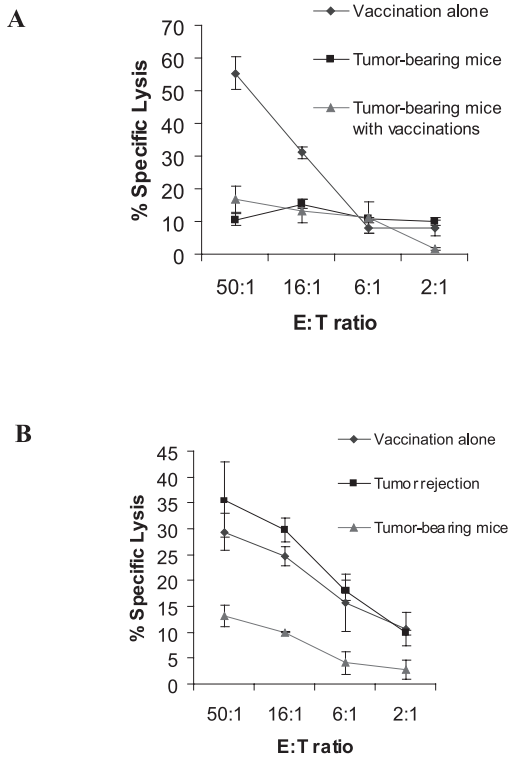

FIGURE 2. Progressive $E7^+$ tumor growth correlates with the late-stage tolerance of tumor-specific $CD8^+$ T cells; rejection of inoculated tumor cells correlates with an effective tumor-specific CTL response. *A*, On day 33, 7 days after the boost, no significant E7-specific cytolytic activity was detected in tumor-bearing mice with or without vaccinations by a standard 6-h in vitro ^{51}Cr-release assay; in contrast, a significant E7-specific cytolytic activity was induced in the control vaccination alone group. The T cell-enriched splenocytes were used as effector cells after in vitro stimulation with $E7_{49-57}$ peptide for 5 days. RMA-E7 cells were used as target cells. It suggests that the established late-stage tumors tolerize E7-specific $CD8^+$ T cells. *B*, After tumor inoculation, in mice with tumor rejection, no established tumors were observed. On day 33 after tumor inoculation, mice with tumor rejection were sacrificed to determine E7-specific CTL response by the ^{51}Cr-release assay. RMA-E7 cells were used as target cells. Tumor-bearing mice and mice with vaccinations alone were used as the controls. In mice with tumor rejection, a high level of E7-specific CTL response was detected, which is comparable to that in mice with vaccinations alone. In tumor-bearing mice, no E7-specific CTL response was detected. Representative data are shown. All of the experiments were repeated three times. RMA-neo cells, RMA-neo cells pulsed with 5 μg/ml LCMV GP_{33-41} peptide, or RMA-neo cells pulsed with 5 μg/ml $E7_{49-57}$ peptide were also used as target cells to confirm E7-specificity of cytolytic activity. Background lysis on RMA-neo cells or RMA-neo cells with GP_{33-41} peptide was $<10\%$ at the highest E:T ratio. Lysis on RMA-neo with $E7_{49-57}$ peptide was similar to lysis on RMA-E7 cells (data not shown).

Rejection of inoculated tumor cells correlates with an effective tumor-specific CTL response

Ten percent of mice did not establish tumors after tumor cell inoculation through day 33 (data not shown). We hypothesized that tumor rejection in these mice resulted from an effective E7-specific CTL response induced by tumor cells. On day 33 after tumor inoculation, splenocytes were isolated from mice with tumor rejection to determine whether functional E7-specific CTLs were induced. Tumor-bearing mice without vaccinations or mice treated with vaccinations alone were used as the controls. As shown in Fig. 2*B*, there was a high level of E7-specific cytolytic activity in mice with tumor rejection, which was comparable to the cytolytic activity induced by vaccinations alone. In contrast, there was no significant cytolytic activity in tumor-bearing mice, as we showed previously. Therefore, progressive tumor growth correlates with the late-stage dysfunction of tumor-specific $CD8^+$ T cells; rejection of inoculated tumor cells correlates with the activation of tumor-specific $CD8^+$ T cells. Previously, other groups also showed that tumor cells activated tumor-specific $CD8^+$ T cells (8, 9, 15, 16). Tumor-specific $CD8^+$ T cells can be activated through cross-presentation by professional APCs that acquire tumor Ags from tumor cells (17, 18). Our data suggest that RMA-E7 tumor cells are able to induce E7-specific CTL response at the early stage; tumor cells are rejected if initial CTL killing outruns the proliferation of tumor cells.

At the late stage of tumor growth, E7 epitope-specific $CD8^+$ T cells are still present in the periphery, and are capable of producing IFN-γ in response to in vitro Ag stimulation

To determine whether the loss of cytolytic activity of tumor-specific $CD8^+$ T cells is due to a deletion of tumor-specific $CD8^+$ T cells, on day 33 after tumor inoculations, we performed an IFN-γ

ELISPOT assay to quantitate $E7_{49-57}$-specific IFN-γ-secreting $CD8^+$ T cells using splenocytes isolated from mice in different groups. As shown in Fig. 3, the frequency of $E7_{49-57}$-specific IFN-γ-secreting $CD8^+$ T cells in tumor-bearing mice with or without vaccinations was similar to that in mice treated with vaccinations alone. At this time point, tumor-bearing mice with or without vaccinations had no E7-specific cytolytic activity, whereas mice treated with vaccinations alone had E7-specific cytolytic activity. Therefore, at the late stage in tumor-bearing mice, although there is no E7-specific cytolytic activity, E7-specific $CD8^+$ T cells are still present in the periphery and can secret IFN-γ in response to Ag stimulation. It suggests that the late-stage nonlytic E7-specific $CD8^+$ T cells are not programmed to die shortly, despite a dysfunctional state. It was reported that anergic $CD8^+$ T cells could persist in vivo in constant numbers for at least 1 year (19).“Split anergy” was reported as a functional state of $CD8^+$ T cells. Otten and Germain (20) demonstrated that a CTL clone was unable to secret IL-2, but retained cytolytic activity in the absence of costimulation. Vezys et al. (21) showed that chronic encounter of Ag by intestinal $CD8^+$ T cells resulted in the loss of IFN-γ secretion function, but the cytolytic activity was retained. It suggests that cytokine production and cytolytic activity can be triggered through independent mechanisms.

The kinetics of cytolytic activity of tumor-specific $CD8^+$ T cells in tumor-bearing mice

We reasoned that tumor cells induced E7-specific CTLs at the early stage; then, therapeutic vaccination amplified the CTL response and delayed tumor growth; however, tumor-specific $CD8^+$ T cells lost cytolytic function at the late stage of tumor growth, which resulted in the failure of further vaccinations. Therefore, to depict the progression of immune dysfunction of $CD8^+$ T cells, the kinetics of E7-specific $CD8^+$ T cell cytolytic activity was examined in tumor-bearing mice. As described above, therapeutic vaccinations on day 12 slowed down the tumor growth, which suggests that tumor-specific $CD8^+$ T cells are functional before day 12 and for a short period after day 12. However, tumor-specific $CD8^+$ T cells lost cytolytic activity by day 33. Thus, we determined E7-specific cytolytic activity from day 12 through day 33 in tumor-bearing mice with therapeutic vaccinations.

The schedule of experiments was shown in Fig. 4*A*, and the data of E7-specific cytolytic activity were shown in Fig. 4*B*. There was no E7-specific cytolytic activity in tumor-bearing mice on day 12. On day 16 (4 days after the first vaccination), there was a detectable level of E7-specific $CD8^+$ T cell cytolytic activity in tumor-bearing mice with therapeutic vaccination, when compared with that in tumor-bearing mice without vaccination or mice treated with vaccination alone. It suggests that tumor cells initially can prime tumor-specific $CD8^+$ T cells in tumor-bearing mice, but it is not high enough to be detected by in vitro ^{51}Cr-release assay at the early stage. Therefore, we did not detect E7-specific cytolytic activity on day 12 in tumor-bearing mice. The primed CTLs were amplified rapidly by the first vaccination, and the CTL response level was high enough to be detected on day 16, although vaccination alone cannot induce a detectable CTL response at this time point. On day 23 (4 days after the second vaccination), there was a high level of E7-specific cytolytic activity in tumor-bearing mice with vaccinations, tumor-bearing mice without vaccinations, and mice only treated with vaccinations. Our data indicate that on day 23 in tumor-bearing mice, cytolytic function of tumor-specific $CD8^+$ T cell is still not impaired and the CTL response can reach to a high level comparable to that induced by vaccinations.

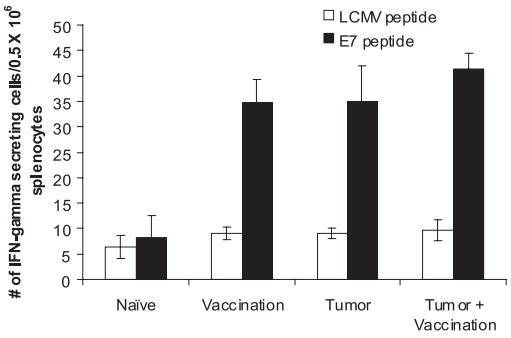

FIGURE 3. Tolerant E7 epitope-specific $CD8^+$ T cells are capable of producing IFN-γ in response to in vitro Ag stimulation. On day 33 after tumor inoculation, splenocytes from four mice per group were pooled, and an IFN-γ ELISPOT assay was used to enumerate $E7_{49-57}$ peptide-specific IFN-γ-secreting cells after in vitro stimulation with $E7_{49-57}$ peptide (■) or the control LCMV GP_{33-41} peptide (□) for 40 h. There were four groups: naive mice, mice treated with vaccinations alone, tumor-bearing mice without vaccinations, and tumor-bearing mice with vaccinations. The numbers of IFN-γ-secreting cells in 0.5×10^6 splenocytes are shown (±SD). The experiment was repeated three times, and representative data are shown.

Freshly isolated RMA-E7 tumor cells from tumor-bearing mice become resistant to E7-specific CTL killing, which is not due to the loss of expression of E7 *gene*

Paradoxically, a progressively growing tumor did induce a high level of CTL response on day 23, but tumor growth was not effectively retarded. To determine whether in vivo growing tumor cells developed a resistance to the killing by functional CTLs, in vitro cultured RMA-E7 tumor cells and freshly isolated in vivo growing tumor cells were used to compare E7-specific killing by functional E7-specific CTLs (Fig. 5*A*). Functional E7-specific CTLs were generated from mice vaccinated with E7 DNA vaccine. Splenocytes from vaccinated mice were in vitro stimulated with $E7_{49-57}$ peptide for 5 days and used as effector cells for in vitro E7-specific cytotoxicity assay. E7-specific CTLs can efficiently kill in vitro cultured RMA-E7 cells; however, the killing activity was significantly decreased for RMA-E7 tumor cells freshly explanted from mice. Both Western blotting analysis (Fig. 5*B*) and RT-PCR assay (N. Obholzer and L. Qiao, unpublished observations) data showed that E7 protein expression in freshly isolated tumor cells was not decreased, but increased. The data suggest that in vivo growing RMA-E7 tumor cells become more resistant to E7-specific CTL killing, which is not due to the loss of expression of *E7* gene.

Discussion

In this report, using a nontransgenic T cell model, we examined the dynamics of tumor-specific $CD8^+$ T cell response to the growing tumor and therapeutic vaccinations and revealed a progression of tumor-specific $CD8^+$ T cell dysfunction during tumor growth, which may explain the mere transient efficacy of CTL-based therapeutic vaccinations. We propose a model to depict the effects that a progressively growing tumor has on tumor-specific $CD8^+$ T cells and therapeutic vaccinations. After tumor cell inoculation, tumor-specific $CD8^+$ T cells can be primed likely through cross-presentation by professional APCs that acquire tumor Ags from tumor cells (17, 18). In mice without an established tumor, a strong CTL response at the initial stage results in rejection of inoculated tumor cells. In tumor-bearing mice, a weak CTL response is normally induced at the early stage, which is not sufficient to lead to tumor rejection. Subsequent therapeutic vaccination induces a more rapid and greater CTL response and eliminates more tumor cells, which slows down tumor growth, whereas in the absence of therapeutic vaccination, the low level of CTL response by tumor-primed

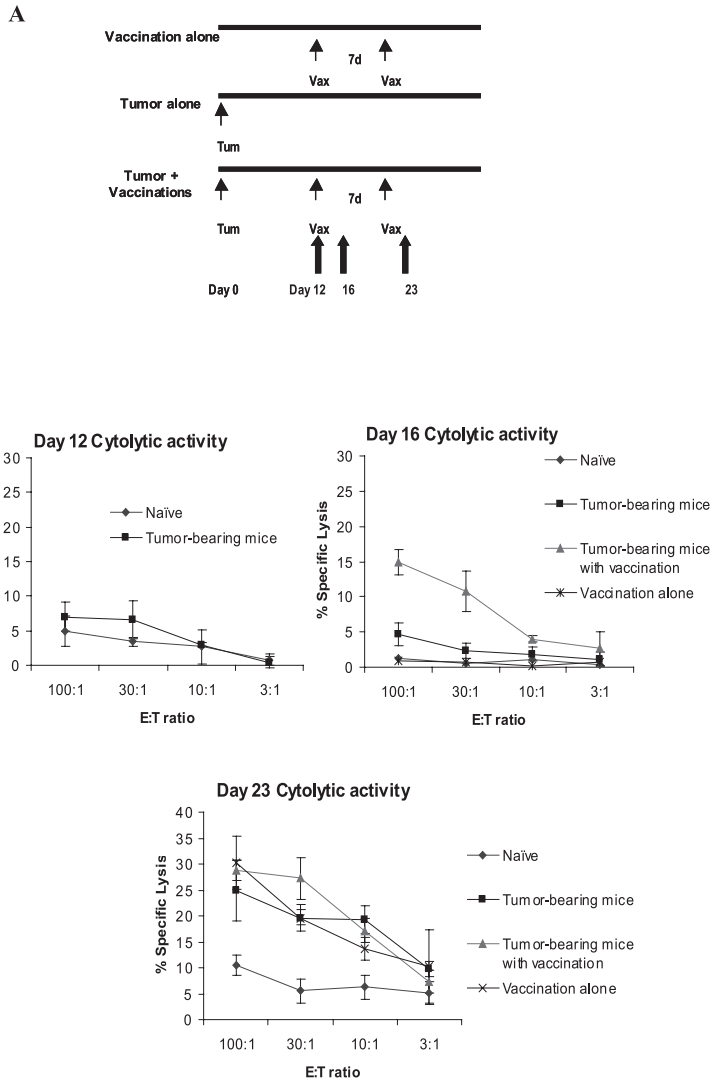

FIGURE 4. The kinetics of E7-specific cytolytic activity during progressive tumor growth. *A*, On day 12 after tumor inoculation, E7-specific cytolytic activity in tumor-bearing mice was determined by a standard 6-h in vitro ^{51}Cr-release assay. In tumor-bearing mice with therapeutic vaccinations, four mice were vaccinated on day 12. On day 16, two mice were used for the ^{51}Cr-release assay. Two remaining mice were boosted on day 19. On day 23, these two mice were used for the ^{51}Cr-release assay. The naive mice, tumor-bearing mice without vaccinations, and mice with vaccinations alone were used as the controls at the corresponding time points. *B*, E7-specific cytolytic activity was determined using splenocytes isolated from mice in different groups. RMA-E7 cells were used as the target cells. On day 12, there was no E7-specific cytolytic activity in naive mice and tumor-bearing mice. On day 16, E7-specific cytolytic activity was detected in tumor-bearing mice with vaccination, but not in tumor-bearing mice or mice treated with vaccination alone. On day 23, there was a high level of E7-specific cytolytic activity in tumor-bearing mice with or without vaccination and mice treated with vaccinations alone. Background lysis on RMA-neo cells or RMA-neo with GP_{33-41} peptide was $<10\%$ at the highest E:T ratio. Lysis on RMA-neo with $E7_{49-57}$ peptide was similar to lysis on RMA-E7 cells (data not shown). All of the experiments were repeated three times, and representative data are shown.

CD8$^+$ T cells cannot efficiently delay tumor growth. When the tumor progressively grows in the absence of therapeutic vaccination, the increase of tumor Ag load induces more tumor-specific CTLs likely through cross-presentation, and tumor-specific CTL response reaches a very high level, even without therapeutic vaccinations. However, in vivo growing tumor cells become more resistant to CTL killing despite functional tumor-specific CTLs present in tumor-bearing mice. The tumor resistance to CTL killing is not due to the loss of tumor Ag gene expression. The tumor Ag protein expression by in vivo growing tumor cells is even increased, which might contribute in part to the increase of tumor-specific CTL response through cross-presentation during progressive tumor growth. At the late stage, tumor-specific CD8$^+$ T cells lose cytolytic function, and the further vaccination cannot induce tumor-specific cytolytic activity. Therefore, both nonlytic tumor-specific CD8$^+$ T cells at the late stage and immune evasion by tumor cells after in vivo growth might contribute to the gradual invalidation of therapeutic vaccinations during progressive tumor growth. Tumor-specific CD8$^+$ T lymphocytes enter the activation phase at the early stage, tumor-specific CTL response reaches a maximal level in the middle stage, and then tumor-specific CD8$^+$ T cells lose cytolytic function at the late stage, which depends on both persistent tumor burden and a time course.

The mechanisms underlying the immune dysfunction of tumor-specific CD8$^+$ T cells remain unclear. There are a number of proposed mechanisms, including the lack of immunologically costimulatory molecules (22–24), immunosuppressive factors present in the tumor milieu such as TGF-β (25, 26) or IL-10 (27, 28), or the Fas ligand expression on tumor cells (29). Our data demonstrate that tumor-specific CD8$^+$ T cells are partially dysfunctional at the late stage in tumor-bearing mice: they lose cytolytic activity while keeping IFN-γ production function. In chronic viral infection, the fate of virus-specific CD8$^+$ T cells is similar to our findings in tumor-specific CD8$^+$ T cells. In HIV-infected patients, HIV-specific CD8$^+$ T cells produce IFN-γ, but are impaired in cytolytic function (30). In murine chronic LCMV infection, the viral persistence results in functional impairment of CD8$^+$ T cells in a hierarchical fashion. Production of IL-2 and in vitro cytolytic capacity are the first functions compromised, followed by the ability to make TNF-α, whereas IFN-γ production is most resistant to functional exhaustion (31). Tumor growth is comparable to chronic viral infections because Ags are persistently presented to CD8$^+$ T cells. Therefore, we speculate that persistent tumor Ag stimulation causes the dysfunction of tumor-specific CD8$^+$ T cells during tumor growth. Kreuwel et al. (32) showed that functional memory CD8$^+$ T cells underwent peripheral tolerance by exogenous administration of soluble peptides, which implies that persistent tumor Ags can induce dysfunction of tumor-specific CD8$^+$ T lymphocytes. Using adoptive transfer of transgenic CD8$^+$ T cells for the male Ag and different amounts of male bone marrow cells

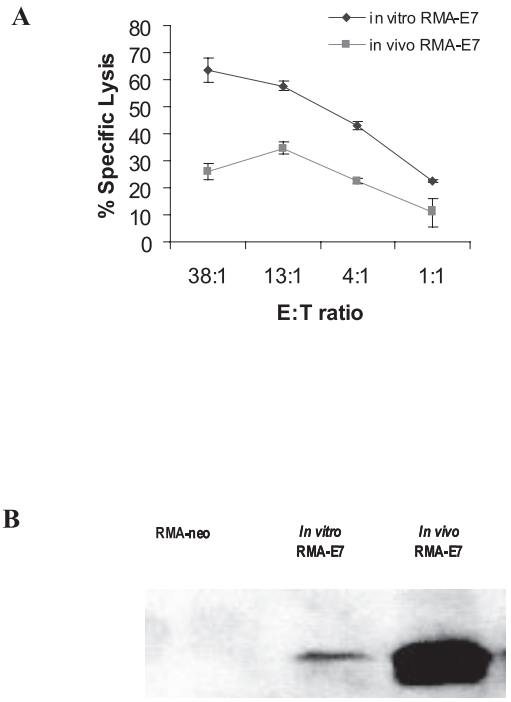

FIGURE 5. In vivo growing RMA-E7 tumor cells become more resistant to E7-specific CTL killing, which is not due to the loss of E7 expression. *A*, Tumor cells explanted from tumor-bearing mice on day 21–30 were compared with in vitro cultured RMA-E7 cells in their susceptibility to E7-specific CTL killing by the ^{51}Cr-release assay. Splenocytes from mice immunized with E7 DNA vaccines were used as E7-specific effector cells after in vitro stimulation with $E7_{49-57}$ peptide for 5 days. In vitro cultured or in vivo growing RMA-E7 cells were used as the target cells. The E:T ratios ranged from 38:1 to 1:1. The killing activity is decreased for in vivo growing RMA-E7 tumor cells when compared with in vitro cultured RMA-E7 cells. Background lysis on RMA-neo cells or RMA-neo with GP_{33-41} peptide was $<10\%$ at the highest E:T ratio (data not shown). *B*, E7 protein in RMA-E7 cells cultured in vitro or growing in vivo was compared. Two hundred seventy micrograms of cell lysate protein was load per sample. RMA-neo cell lysate was used as the negative control. The E7 protein level was much higher in RMA-E7 tumor cells explanted from tumor-bearing mice compared with in vitro cultured RMA-E7 cells. E7 protein was detected at a molecular mass equivalent of 21 kDa. All of the experiments were repeated more than three times, and representative data are shown.

into female mice, Tanchot et al. (33) showed that a lower amount of male bone marrow cells were eliminated by activation of $CD8^+$ T cells; a higher amount of male bone marrow cells induced $CD8^+$ T cell tolerance, and both $CD8^+$ T cells and male cells persisted in the periphery. It was proposed that tolerance was a consequence of Ag persistence and an excessive Ag load (33). The Ag-induced nonresponsiveness (34–36) might be a generic consequence for T cells after prolonged antigenic stimulation in different experimental systems.

The understanding of the kinetics of tumor-specific CTL response is important to improve the vaccination strategies against tumors. The functional state of tumor-specific $CD8^+$ T cells decides the efficacy of therapeutic vaccination. The tumor itself can turn on, and then turn off tumor-specific CTL response at different stages during progressive tumor growth. Therefore, it is important to design a vaccination regimen according to the stages of tumor growth. The early stage therapeutic vaccination will be effective when tumor-specific T cells are functional. The late-stage therapeutic vaccination will fail due to immune dysfunction of tumor-specific $CD8^+$ T cells, and a more potent immunotherapeutic strategy to reverse the functional deficits in vivo is required. Surgical removal of the tumor mass will drastically reduce the tumor cell load. It should be promising to eliminate the remaining tumor cells if an effective tumor-specific CTL response can be induced timely and strongly.

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Disclosures

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