Generation of Antitumor Immunity by Cytotoxic T Lymphocyte Epitope Peptide Vaccination, CpG-oligodeoxynucleotide Adjuvant, and CTLA-4 Blockade

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ABSTRACT

Although peptide immunization often leads to the induction of strong T-cell responses, it is seldom effective against established tumors. One possibility is that these T-cell responses are not strong enough or do not last sufficiently long to have an effect in tumor eradication. Here, we examined the role of synthetic oligodeoxynucleotide (ODN) adjuvants containing unmethylated cytosine-guanine motifs (CpG-ODN) and CTLA-4 blockade in enhancing the antitumor effectiveness of peptide vaccines intended to elicit CTL responses. The results show that combination immunotherapy consisting of vaccination with a synthetic peptide corresponding to an immunodominant CTL epitope derived from tyrosinase-related protein-2 administered with CpG-ODN adjuvant and followed by systemic injection of anti-CTLA-4 antibodies increased the survival of mice against the poorly immunogenic B16 melanoma. Interestingly, whereas this combination therapy was effective when administered to tumor-bearing mice (therapeutic protocol), it had no significant effect when applied in the prophylactic mode (i.e., before the tumor challenge). Moreover, the antitumor effect of the combination immunotherapy required the participation of CD4+ and CD8+ T lymphocytes and was accompanied by the induction of antitumor CD4+ T-cell responses. The overall results suggest that peptide vaccination of tumor-bearing mice, applied in combination with a strong adjuvant and CTLA-4 blockade, is capable of eliciting durable antitumor T cell responses that provide survival benefit. These findings bear clinical significance for the design of peptide-based therapeutic vaccines for human cancer patients.

INTRODUCTION

Successful T-cell-based immunotherapy leading to increased survival of cancer patients is largely dependent upon the appropriate activation and persistence of antigen-specific antitumor T-cell responses. CD8+ CTLs constitute one of the most important arms of the immune system, exhibiting the capacity of recognizing and destroying cancerous cells. CTLs recognize antigen on tumor cells in the form of peptide fragments complexed with MHC class I molecules (1, 2). These T-cell peptide epitopes are derived from TAAs, which correspond to proteins that are synthesized and subsequently processed by tumor cells. With the continued identification of TAAs and their corresponding T-cell epitopes (3), major interests have been kindled for the development of peptide-based vaccines intended to induce antitumor immunity. Indeed, the use of synthetic peptide-based vaccines for the induction of CTL responses with the goal of providing antitumor effects has been extensively researched (4). However, in most instances, artificial tumor model systems have been used, which are based on peptide vaccines prepared from “surrogate TAAs” such as viral or foreign proteins selected for their high immunogenic nature. Although these tumor model systems have served as proofs of principle for understanding some of the mechanisms involved in the generation of in vivo CTL responses bearing “antitumor” effector function, the findings of these studies have not taken in consideration some of the complexities inherent to the use of natural TAAs as immunogens. Specifically, because most TAAs are also expressed to some extent on some normal tissues, the significant barrier posed by immune tolerance has not been fully addressed.

In addition to the above, other hurdles have hindered the development of synthetic peptide-based vaccines aimed at inducing CTL responses (5). First, peptides on their own are seldom immunogenic and therefore must routinely be coadministered with immune adjuvants whose function is to provide the necessary alarm or danger signals to awaken the immune system (6, 7). Most disturbingly, in some cases, peptide vaccination, even in the presence of adjuvant, can lead to the elimination of the CTLs recognizing the specific peptide epitope, resulting in enhanced tumor growth (8, 9). Based on these observations, it has been assumed that to activate the naïve CTLs into effector killer cells, the peptide in the vaccine has to be presented by activated and licensed DCs (10–12). On the other hand, presentation of peptide epitopes by nonprofessional APCs or unlicensed DCs may result in the induction of CTL anergy or even in their demise. To surmount these obstacles, some researchers have opted to use peptide-pulsed DCs as vaccines with the purpose of inducing antitumor CTL responses (13, 14). Although this approach can result in effective antitumor immunity both in animal models and in human clinical studies (13–16), the complex nature of this labor-intensive therapeutic approach curtails its widespread use in the general cancer patient population.

In previous studies, we observed that peptide or protein vaccination administered with immunostimulatory ODNs containing unmethylated cytosine-guanine (CpG) motifs as adjuvant significantly increased the number of antigen-specific CTLs resulting in antitumor immunity (17). The adjuvanticity of CpG-ODN on CTL responses was ascribed to its capacity to stimulate and increase the numbers of activated DCs and to the antiapoptotic effects that this compound had on T lymphocytes, which allowed the expansion of antigen-stimulated cells by preventing activation-induced cell death (18, 19). In these studies, we used chicken Ova and its immunodominant CTL epitope (Ova257–264) as model TAA, and thus, these experiments did not realistically represent the scenario of a natural TAA that must follow the rules of T-cell tolerance. With the objective of extending our previous findings to a more physiological TAA, we have now selected the well-characterized melanoma-specific, H-2Kb-restricted CTL epitope derived from mouse TRP2 represented by peptide TRP2180–188 (20, 21). In addition, in the work described here, we included a CTLA-4 blockade regimen to prevent down-regulation of the CTL responses due to the cross-linking of CTLA-4 on T cells by its ligands, B7.1 and B7.2, which is known to provide negative signals to the T cells (22–24). Our results show that TRP2180–188 peptide vaccination administered with CpG-ODN adjuvant and CTLA-4 blockade generated significant antigen-specific CD8+ CTL responses. Moreover, the vaccination protocol enhanced the overall survival against B16 melanoma when administered in the therapeutic mode but not with a prophylactic protocol. The antitumor effect of this...
peptide vaccine combination required the presence of CD4+ T cells, some of which responded to antigens expressed by the tumor cells. These findings indicate that peptide vaccines may be effective as therapies against established tumors if they are administered in combination with strong adjuvants and immunoregulatory strategies such as CTLA-4 blockade.

MATERIALS AND METHODS

Mice. Female 6–8-week-old C57BL/6, CD4−/−, and CD8−/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). The Mayo Clinic’s Institutional Animal Care and Use Committee approved all experimental protocols described here.

Immunogens, Synthetic Peptides, ODNs, and Antibodies. The TRP2180-188 CTL epitope (SYVYDFFVWL) and the HTL pan DR epitope, known as PADRE (AKKVAAWKLAAA where X = cyclohexylalanine; Ref. 25), were prepared by standard solid-phase methods using an Applied Biosystems synthesizer (Foster City, CA) and purified by high-performance liquid chromatography. The purity (95%) and identity of peptides were confirmed by high-performance liquid chromatography and mass spectrometry analysis. The previously described immunostimulatory synthetic ODN-1826 (17, 18, 26), containing two CpG motifs (TCCATGACGTTCCTGACGTT), was used throughout this work. Synthetic ODNs were prepared with a nuclease-resistant phosphorothioate backbone by the Mayo Molecular Core Facility. Synthesized ODN-CpG was dried under vacuum, resuspended in RNAse, Dnase-free distilled H2O (pH 7.0), and stored at 4°C. Using a lymphotoxin amide-diffusion technique, synthetic ODNs prepared in this manner were found to have less than 0.5 endotoxin unit/µg compound (the usual daily dose). The anti-CTLA-4 antibodies were generated from the UC10−12F10 hybridoma (27) and delivered i.p. after peptide immunization.

Cell Lines. The B16 melanoma and the EL4 thymoma (both H-2b) were obtained from American Type Culture Collection (Manassas, VA). These cell lines were maintained in tissue culture in DMEM containing 10% fetal bovine serum, t-glutamine, and antibiotics. Cell lines used for these experiments were found to be free of Mycoplasma infection.

Immunizations and CpG-ODN Therapy. All experiments were routinely performed in groups of 4–10 mice each. Mice received nine daily s.c. injections of 100 µg of CpG-ODN (referred to as 9X CpG-ODN therapy), at the nape of the neck. On day 5 (in the middle of 9X CpG-ODN therapy), the mice were vaccinated at a proximal site in the nape of the neck with 100 µg of TRP2180-188 peptide emulsified in IFA in a total volume of 100 µl. Where indicated, in some experiments the mice also received 140 µg of PADRE peptide mixed in the IFA emulsion to activate nonspecific T-helper responses. CTLA-4 blockade consisted of the i.p. injection of 100 µg of purified anti-CTLA-4 antibody on day 6 (1 day after the peptide administration), followed by two injections of 50 µg each of antibody applied on days 8 and 10. The peptide vaccine was administered either 7 days before tumor challenge (prophylactic protocol) or 7 days after the injection of the live tumor cells (therapeutic protocol). A schematic representation of the prophylactic immunization protocol is shown in Fig. 1A.

Evaluation of Antitumor Effects in Vaccinated Mice. The effect of peptide vaccination (with and without 9X CpG) on tumor growth and survival was evaluated in both prophylactic and therapeutic modes. For prophylaxis, the mice were immunized with TRP2180-188 peptide as described above, and 7 days later, they were challenged s.c. with 5 × 104 live B16 melanoma cells in the rear leg flank. For therapeutic evaluation, the mice received the TRP2180-188 peptide injection 7 days after the tumor challenge. Mice were observed daily or every other day, and when tumors became evident, their perpendicular diameters were measured using a set of calipers. Although some tumor-bearing mice died on their own, most animals were euthanized when tumors became ulcerated or surpassed 100 mm² in size (as required by the Institutional Animal Care and Use Committee). Statistical analyses for evaluating the survival advantage of the vaccines were performed using log-rank tests.

CTL Assays. LNs from immunized mice were removed 7 days after the peptide injection or otherwise as indicated, and single-cell suspensions were obtained by teasing the organs through a sterile nylon mesh. In most experiments, cells were pooled from each group of mice. LN cells (5 × 106) from immunized mice were maintained for 5 days in a humidified 7% CO2 incubator at 37°C in IMDM containing 10% fetal bovine serum (complete IMDM) and 25 IU interleukin 2/ml in 24-well flat-bottomed plates. The cytotoxic activity of the cells was determined by 4-h 51Cr release assays. Peptide-pulsed target cells were prepared by incubating EL4 cells with 10 µg/ml peptide in tissue culture for 12–16 h. B16 melanoma tumor cells were treated with 100 IU/ml recombinant mouse IFN-γ for 18–24 h before the cytotoxicity assays to increase the expression of MHC class I and adhesion molecules. Approximately 3 × 105 target cells (EL4, peptide-EL4, or B16 cells) were labeled with 200 µCi of Na3Cr (Amersham Pharmacia Biotech, Piscataway, NJ) for 90 min at 37°C. Target cells were washed extensively and mixed with various numbers of effectors in 96-well round-bottomed plates. After a 4-h incubation period at 37°C, the radioactivity in the supernatants was determined using a TopCount scintillation counter (Packard Instruments, Meriden, CT), and the percentage of specific lysis was defined by the formula [(experimental 51Cr release − spontaneous 51Cr release) / (maximum 51Cr release − spontaneous 51Cr release)], where spontaneous release was the radioactivity of target cells released into the supernatant in the absence of effector cells (background), and maximum release was the radioactivity released by the targets incubated with 0.1% Triton X-100. All experimental determinations were performed in triplicate, and the averages and SD were consistently below 15% of the value of the mean.

Other Functional T-cell Assays. The response of T cells to antigen stimulation was also evaluated by the production of lymphokines induced by antigen stimulation. Numbers of IFN-γ-producing T lymphocytes were determined by flow cytometry using intracellular staining with fluoresceinated monoclonal antibodies specific for IFN-γ (BD-PharMingen, San Diego, CA). For these experiments, 5 × 104 LN cells were cultured in 1 ml of complete IMDM for 5 days in the presence of 50 units of interleukin 2/ml. After this time, the cells were stimulated for 12 h with an equal number of EL4, peptide-pulsed EL4, or B16 cells (treated with 100 units of IFN-γ 24 h before use). For some experiments (Fig. 8), the LN cells were stimulated with B16 melanoma cell lysates (3 freeze/thaw cycles), with the intention of stimulating the T cells via the cross-presentation of antigen by the APCs in the LN cell preparation. The stimulated LN cells were harvested and labeled for surface CD8 (or CD4) and intracellular IFN-γ using FITC-labeled specific monoclonal antibodies (PharMingen) following the detailed instructions provided by the vendor. Cells were analyzed on a Becton Dickinson FACScan (San Jose, CA).
RESULTS

Inefficacy of Peptide Vaccination for Protecting against a Melanoma Challenge. The antitumor effect of peptide vaccination combined with CpG-ODN adjuvant and CTLA-4 blockade was first examined in the prophylactic setting (before tumor challenge). For these experiments, we selected the immunodominant H-2K^d-restricted TRP2_180-188 CTL epitope, which was previously reported to confer resistance against B16 melanoma challenge in mice vaccinated with peptide-pulsed DCs but not in mice vaccinated with peptide emulsified in IFA (21). Groups of five mice received one of various vaccination protocols shown in Fig. 1A, and 7 days after peptide injection, the animals were challenged via s.c. route with $4 \times 10^4$ live B16 melanoma cells. The data presented in Fig. 1B indicate that vaccination with CTL peptide TRP2_180-188 together with the T-helper epitope PADRE, using 9XCpG-ODN adjuvant, even with the addition of CTLA-4 blockade, failed to provide a survival advantage against the subsequent tumor challenge. These results contrast with our previous observations in the Ova system, where peptide vaccination with 9XCpG-ODN adjuvant and without the need of CTLA-4 blockade protected the mice (80% survival) against a challenge with B16 cells expressing Ova (17).

Induction of Antitumor CTL Responses by Peptide Vaccination Requires CpG-ODN Adjuvant. The failure to protect mice against a B16 melanoma challenge using the above vaccination protocols could be explained if TRP2_180-188-specific CTL responses were not generated by these vaccines. Thus, we assessed whether antigen-specific, tumor-reactive CTLs could be induced after a single immunization with the TRP2_180-188 peptide/9XCpG-ODN vaccine. Significantly higher CTL responses to both peptide-pulsed EL4 and B16 melanoma were generated in the mice that received TRP2_180-188 peptide in IFA and 9XCpG-ODN, as compared with those mice that were vaccinated with the peptide in IFA alone (Fig. 2). Furthermore, the addition of CTLA-4 blockade to the vaccination protocol slightly increased the magnitude of the response to the B16 melanoma target cells (Fig. 2, right panel). These results demonstrate that CpG-ODN adjuvant was required for the induction of CTL responses against a peptide-based vaccine derived from a TAA, which is similar to what we reported in the Ova system (17). Most important, these findings indicate that even though melanoma-reactive CTLs were generated with the TRP2_180-188/9XCpG-ODN vaccine, these responses were not sufficiently robust to protect the mice against a tumor challenge.

Effects of Peptide Vaccination in Tumor-bearing Mice. The TRP2_180-188/9XCpG-ODN vaccine was also evaluated in the therapeutic mode (i.e., in tumor-bearing mice). The rationale for carrying out these experiments was the possibility that an initial CTL response in tumor-bearing animals could result in the generation of additional antitumor CTLs and even possibly helper T-cell activities against melanoma antigens via epitope spreading and the cross-presentation of antigens. Thus, we compared the antitumor effectiveness of TRP2_180-188/9XCpG-ODN vaccination against B16 melanoma in the therapeutic and prophylactic settings. The results of this experiment indicated that the TRP2_180-188/9XCpG-ODN vaccine combined with CTLA-4 blockade significantly increased the survival time (~50 days) of mice against B16 melanoma, but only when administered in the therapeutic mode (Fig. 3). In separate experiments, we established that all three components of the therapeutic vaccine (TRP2_180-188 peptide, 9XCpG-ODN, and CTLA-4 blockade) were required to achieve the survival advantage and that the addition of PADRE HTL epitope did not increase the therapeutic benefit of the vaccine (data not shown). These results support our hypothesis that joint administration of a CTL peptide with CpG-ODN adjuvant and CTLA-4 blockade during the course of tumor progression results in enhanced antitumor activity.

Although the therapeutic vaccination increased the survival time against B16 melanoma, no complete tumor rejections were observed, and all animals ultimately succumbed to the disease. This could be due to: (a) the rapid growth of this tumor, which may not allow the sufficient time to generate an effective immune response; and/or (b) the induction of anergy or other immune escape-suppressor mechanisms by the tumor as it progresses in the host. These possibilities were examined by combining the prophylactic and therapeutic vaccination protocols, which would allow a more speedy generation of CTLs that supposedly would trigger the cascade of events leading to tumor rejection. The experiment presented in Fig. 4 demonstrates that,
Fig. 4. Enhancement of antitumor effect of TRP2180–188 peptide vaccination by prophylactic/therapeutic combination. Age-matched normal C57BL/6 mice were vaccinated in the prophylactic mode (left panel) with TRP2180–188 Peptide/9XCpG-ODN and CTLA-4 blockade (○), TRP2180–188 Peptide in IFA alone (□), or 9XCpG and CTLA-4 blockade, but no peptide (▲). Additionally, one group of mice vaccinated with TRP2180–188 peptide/9XCpG-ODN with CTLA-4 blockade received a second complete regimen of vaccination after tumor challenge (■). As a control, one group of mice received a prophylactic/therapeutic vaccine combination of 9XCpG-ODN with CTLA-4 blockade without TRP2180–188 peptide (△). For comparison, mice were vaccinated in the therapeutic mode (right panel) with TRP2180–188 Peptide/9XCpG-ODN with CTLA-4 blockade (○), TRP2180–188 Peptide in IFA alone (□), or 9XCpG and CTLA-4 blockade but no peptide (▲). Vaccines used in these experiments did not include the PADRE HTL epitope. The data were analyzed using log-rank tests.

Indeed, the majority (80%) of mice receiving two subsequent vaccination procedures (one prophylactic and the other therapeutic) survived the B16 melanoma challenge, whereas, as noted previously, the prophylactic vaccination alone had no effect, and the therapeutic vaccine extended survival but did not cure the mice. In subsequent experiments, we determined that all three components of the prophylactic/therapeutic combination vaccine (TRP2180–188 peptide, 9XCpG-ODN, and CTLA-4 blockade) were required during both immunizations to attain the antitumor effects (data not shown). The immunity provided by the combination prophylactic/therapeutic vaccine was long-lasting, as determined by the ability of 100% of the surviving mice from this experiment to reject a second tumor challenge (data not shown). These results indicate that the double immunization protocol may be more effective than the therapeutic vaccine because of the expedient generation of an immune response against the rapidly growing B16 melanoma. Moreover, the results also suggest that the inability of the therapeutic vaccine to eradicate disease is probably not solely due to an immune inhibitory activity of the tumor.

Effects of Peptide Vaccination in Tumor Growth Kinetics. The antitumor activity of the TRP2180–188/9XCpG-ODN vaccine with CTLA-4 blockade therapy was also evaluated by the measurement of tumor sizes in the various groups of mice. The analysis of tumor growth kinetics (Fig. 5) indicated that the tumors in mice receiving the complete therapeutic vaccination protocol (Fig. 5, ▲) grew much slower than the tumors in mice that received the prophylactic vaccine (Fig. 5, ◇) or those animals that were left untreated (Fig. 5, ●). Furthermore, the tumors also grew slowly in the small number of mice that developed tumors when receiving the combination of prophylactic/therapeutic vaccine (in this experiment, 3 of 10 mice developed tumors; Fig. 5, ●).

Requirement of CD4+ and CD8+ T Cells for Antitumor Immunity. The roles of CD8+ CTLs and CD4+ T-helper lymphocytes in providing survival advantage of mice to B16 melanoma via the TRP2180–188/9XCpG-ODN vaccination with CTLA-4 blockade were examined. Various modes of vaccination were applied to wild-type C57BL6 mice and to CD8- and CD4-deficient T (−/−) mice (both in the C57BL6 background), and their survival to a B16 melanoma challenge was evaluated. As shown in Fig. 6, the survival advantage conferred by the therapeutic and the prophylactic/therapeutic vaccines was not evident in either CD4−/− or CD8−/− mice, indicating that both CD4+ and CD8+ T cells were required for the antitumor effects of the vaccine.

Although the requirement of the CD8+ CTLs was expected, the role of CD4+ T-helper lymphocytes in the effectiveness of the TRP2180–188/9XCpG-ODN vaccine is not so clear. In some cases, CD4+ T cells are required for the induction of CTLs, whereas in some circumstances, helper T cells can also play a critical role in the maintenance and longevity of the CTL responses. Thus, we evaluated whether CD4+ T cells were required for the induction of TRP2180–188-specific CTL responses to the 9XCpG vaccine. The results in Fig. 7 indicate that both C57BL6 and CD4−/− mice were capable of producing significant CD8+ T-cell responses to peptide-pulsed EL4 and B16 melanoma cells. Furthermore, the magnitude of the antigen-specific CD8+ T-cell responses induced by the peptide vaccine was not significantly different between the wild-type C57BL6 and the CD4−/− mice. These results suggest that in this model system of antitumor peptide vaccination, CD4+ T cells may be more important for maintenance of CTL responses than for their induction.

The results presented thus far indicate that the therapeutic and the prophylactic/therapeutic vaccines are more effective than the administration of prophylactic vaccine alone because they are capable of inducing an antitumor CD4+ T-cell response, which could be critical for the persistence of the CTL responses. The results shown in Fig. 8, where antigen-specific CD8+ and CD4+ T-cell responses were measured in vaccinated tumor-bearing mice, agree with this assessment. First, mice that received the therapeutic or the prophylactic/therapeutic vaccine displayed significant CD4+ responses to B16 melanoma antigens as compared with untreated or prophylactic-vaccinated mice. Second, CD8+ T-cell responses were also significantly higher in the same groups of mice exhibiting melanoma-reactive CD4+ T-cell responses. Third, and most important, no significant CD8+ T-cell responses to either peptide TRP2180–188 or B16 melanoma were evident in the prophylactic-vaccinated tumor-bearing mice. Because 9XCpG-ODN induces marked lymphadenopathy, especially in the draining LNs (19), we proceeded to reanalyze the data from the experiment presented in Fig. 8 to evaluate the actual frequency of effector T cells (antigen-reactive lymphokine secreting CD4+ or CD8+ T lymphocytes) in the LNs of the vaccinated mice. The results presented in Fig. 9 indicate that approximately 20–30% of all CD8+ T cells in the LNs from tumor-bearing mice receiving the prophylactic/therapeutic or therapeutic vaccines were...
Fig. 6. An antitumor effect of peptide vaccination requires both CD4+ and CD8+ T lymphocytes. Normal wild-type (WT) C57BL/6, BL/6-CD8-/-, and BL/6-CD4-/- mice received TRP2180-188 peptide/9XCpG-ODN with CTLA-4 blockade in the prophylactic mode (□), therapeutic mode (○), or the prophylactic/therapeutic combination protocol (□). As controls, one group of mice was left untreated (○). Vaccines used in these experiments did not include the PADRE HTL epitope. The data were analyzed using log-rank tests between the control groups and the various treatment groups.

The overall results demonstrate that prophylactic vaccination was efficient at inducing CD8+ T-cell responses in tumor-free animals (Figs. 2 and 7) but that these responses may have been down-regulated in the tumor-bearing mice, possibly due to the absence of CD4+ tumor-reactive T cells, which are only induced by the therapeutic vaccination.

**DISCUSSION**

The generation of antitumor CTL responses is an attractive approach for the treatment or prevention of cancer. Many types of T-cell-inducing vaccines ranging from gene-modified tumor cells to antigen-pulsed DCs have shown significant antitumor effects both in human and in mouse model systems (13–16, 28, 29). In addition, manipulation of the immune system via costimulation or CTLA-4 blockade can dramatically improve the effectiveness of some of these vaccines (22, 23, 30, 31). Recently, the identification of the TAA-derived CTL epitopes has opened the door to the development of highly defined vaccines in the form of in vivo-administered synthetic peptides that seek to elicit antitumor CTL responses. Although synthetic peptides are probably one of the simplest types of vaccines to prepare and characterize, these compounds are usually not very immunogenic unless they are administered in combination with strong adjuvants, which provide the appropriate danger/activation signals to the immune system. We reported previously (17) that vaccination of normal mice using the immunodominant and supposedly “strong” CTL epitope Ova257–264 (SIINFEKL) as a synthetic peptide formulated in an oil:water emulsion (IFA) failed to induce CTL responses unless the animals also received CpG-ODN as adjuvant. A possible explanation for these results is that our mouse colony is close to being germ-free, which would place the animal’s immune system in a low level of alert with insufficient activated APCs to stimulate CTL responses against an innocuous peptide. The potent adjuvanticity of CpG-ODN for CTL responses is derived from two of its biological properties. First, it is widely known that various forms of CpG-ODN, which mimic bacterial DNA, are able to stimulate and activate APCs such as DCs through their interaction with Toll-like receptor-9 (32–37). Second, as we have recently shown (19), repeated administration of CpG-ODN results in the generation of strong ant apoptotic activity in both activated and naïve T cells via the enhanced expression of bcl-xL and c-FLIP, which would reduce the down-regulation of immune responses due to activation-induced cell death.

Our previous studies demonstrated that the CTL responses to the Ova257–264 Peptide vaccine in the presence 9XCpG-ODN were sufi-
against TRP2 180

Methods

The number of CD4+ T-cell responses, which correlate with the persist-
ence of antigen-specific CD8+ T cells. Groups of four mice were immun-
ized with TRP2180-188 and CTLA-4 blockade in the prophylactic
mode (Thx, □), therapeutic mode (Thx, △), or the prophylactic/therapeutic
combination (Phx, ◦). As a negative control, one group of mice received
the prophylactic protocol, but no TRP2180-188 peptide (Phx, △). Seven days (for
Thx and Phx + Thx) or 21 days (for Phx) after last peptide injection, cells from
draining LNs were harvested and processed as described in “Materials and
Methods” to determine antigen-specific CD4+ and CD8+ T-cell activity
against TRP2180-188 pulsed EL4 cells and B16 melanoma cells. Antigen re-
sponses were determined via flow cytometry analysis for intracellular content
of IFN-γ. The total number of antigen-specific CD4+ and CD8+ T cells/draining
LN was determined by multiplying the percentage of IFN-γ+CD4+ or IFN-
γ+CD8+ cells by the total number of LN cells and subtracting this value from
the number of CD4+ or CD8+ T cells responding nonspecifically to EL4 cells
(without peptide). Horizontal lines represent the mean values for each group.
The data were analyzed using Student's t test, and significance is expressed by
Ps observed between different groups.

CpG ADJUVANT AND CTLA-4 BLOCKADE FOR PEPTIDE-BASED VACCINES

Fig. 8. Therapeutic and prophylactic/therapeutic TRP2180-188/9X CpG-ODN
vaccination in tumor-bearing mice followed by CTLA-4 blockade generates
melanoma-reactive CD4+ T-cell responses, which correlate with the persist-
ence of antigen-specific CD8+ T cells. Groups of four mice were immun-
ized with TRP2180-188/9X CpG-ODN and CTLA-4 blockade in the prophylactic
mode (Phx, ○), therapeutic mode (Thx, △), or the prophylactic/therapeutic
combination (Phx, ◦). As a negative control, one group of mice received
the prophylactic protocol, but no TRP2180-188 peptide (Phx, △). Seven days (for
Thx and Phx + Thx) or 21 days (for Phx) after last peptide injection, cells from
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(without peptide). Horizontal lines represent the mean values for each group.
The data were analyzed using Student's t test, and significance is expressed by
Ps observed between different groups.

Fig. 9. Frequency of antigen-reactive effector T lymphocytes in vaccinated tumor-
bearing mice. The data from Fig. 8 were reanalyzed, and results are presented as the
frequency (percentage) of effector CD8+ or CD4+ T cells (lymphokine-producing)
corresponding to the total CD8+ or CD4+ LN cells, respectively. CD8/EL4p, CD8+ T
cells tested with EL4 cells with TRP2180-188 peptide; CD8/EL4, CD8+ T cells tested
with EL4 cells without peptide; CD8/B16m, CD8+ T cells tested with B16 melanoma cells;
CD4/B16m, CD4+ T cells tested with B16 melanoma cells; CD4/EL4p, CD4+ T cells
tested with B16 melanoma cells. Asterisks denote statistically significant differences
(P < 0.05) in specific groups as compared with their corresponding controls: CD8/EL4p
and CD8+/B16m versus CD8/EL4; and CD4/B16m versus CD4/EL4p.

vaccination was followed by a second round of vaccine, administered
after the tumor challenge (prophylactic/therapeutic protocol), the anti-
tumor effectiveness of this procedure became comparable to that of
the prophylactic Ova vaccine (~80% complete protection for both
systems).

Notably, the two vaccination protocols that provided a survival
advantage (the therapeutic vaccine and the prophylactic/therapeutic
combination) resulted in the generation of CD4+ T-helper responses to
B16 melanoma antigens (Fig. 8). In addition, both of these vacci-
nation protocols failed to protect mice lacking CD4+ T cells (Fig. 6),
indicating that this T-cell subset plays an important role in the anti-
tumor effect of the peptide-based vaccine. Although CD4+ T cells
were not required for the induction of the CTL responses to
TRP2180-188 and B16 melanoma (Fig. 7), the helper T cells appeared
to facilitate the persistence of high numbers of CTLs in tumor-bearing
mice (Figs. 8 and 9). The overall results indicate that with peptide-
based vaccines, the melanoma-reactive CD4+ T-helper cells are
extremely important during the effector phase of the immune re-
sponse, possibly because they allow the CTLs to persist and expand at
the tumor site. In agreement with this hypothesis, we have recently
reported in an in vitro human melanoma system that activated CD4+
T lymphocytes were capable of providing direct costimulatory activity
to a low number of tumor-reactive CTLs, enabling these cells to
rapidly expand in the presence of large numbers of melanoma cells
and ultimately eliminate all of the tumor cells from the cell cultures
(38). The costimulatory function of CD4+ T cells in this system
required cell-to-cell contact with the CTLs and appeared to be medi-
ated via CD27/CD70 and 4-1BB/4-1BBL interactions. It has also
been described both in humans and in mice that adoptively transferred
CD8+ CTLs are more likely to persist and remain functional when
coadministered with antigen-specific CD4+ T cells (39, 40).

The mechanism by which the antitumor CD4+ T-cell responses were
induced by the therapeutic and prophylactic/therapeutic vaccinations
may be through the cross-presentation of melanoma-
derived antigens. It is possible that through the cytolytic action of the
TRP2180-188-specific CTLs, B16 melanoma antigens (or apoptotic
tumor cells) are generated and subsequently captured by DCs, which
become highly activated through the action of the CpG-ODN and then
become highly effective in stimulating melanoma antigen-reactive
CD4+ T-cell responses. The nature of the antigens recognized by the
CD4+ melanoma-reactive T cells is unknown and presently being
investigated.
The current findings hold significant implications for the design of peptide-based vaccination strategies for cancer in human patients. First, it is clear that peptide vaccination alone (in saline) or with simple adjuvants such as IFA will likely not provide a sufficiently strong CTL response to have a clinical benefit. Thus, stronger adjuvants such as CpG-ODN will be required to properly activate APCs and induce stronger T-cell responses. Second, the induction of tumor antigen-reactive CD4+ T-cell responses may be critical for the overall effectiveness of the antitumor CTLs. Although the CD4+ H11001 shown here and in several other systems, enhances the effectiveness of designing approaches to enhance immune responses by preventing negative signals from various human TAAs should facilitate the implementation of this practice in future clinical studies (41–49). Third and last, additional manipulation of the antitumor immune response may be required to prevent the down-modulation of T-cell responses so these can last sufficiently long to curb tumor progression. One of the most promising approaches to enhance immune responses by preventing negative signals to T lymphocytes is the use of CTLA-4 blockade, which, as shown here and in several other systems, enhances the effectiveness of antitumor vaccines. We hope that in the near future, clinical studies in human patients may be able to put into practice the current findings to demonstrate that peptide-based vaccines indeed constitute a simple, effective, and hopefully less hazardous approach than chemotherapy and radiation therapy for the treatment and prevention of cancer.

REFERENCES


Generation of Antitumor Immunity by Cytotoxic T Lymphocyte Epitope Peptide Vaccination, CpG-oligodeoxynucleotide Adjuvant, and CTLA-4 Blockade

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