Optimized Peptide Vaccines Eliciting Extensive CD8 T-Cell Responses with Therapeutic Antitumor Effects

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Abstract

A major challenge for developing effective therapeutic vaccines against cancer is overcoming immunologic tolerance to tumor-associated antigens that are expressed on both malignant cells and normal tissues. Herein, we describe a novel vaccination approach, TriVax, that uses synthetic peptides representing CD8 T-cell epitopes, Toll-like receptor agonists that function as potent immunologic adjuvants and costimulatory anti-CD40 antibodies to generate large numbers of high-avidity antigen-reactive T cells capable of recognizing and killing tumor cells. Our results show that TriVax induced huge numbers of long-lasting antigen-specific CD8 T cells that displayed significant antitumor effects in vivo. The administration of a TriVax formulation containing a CD8 T-cell epitope derived from a melanosomal antigen (Trp2180-188) elicited antigen-specific CD8 T cells that induced systemic autoimmunity (vitiligo). More important, TriVax immunization was effective in eliciting potent protective antitumor immunity as well as remarkable therapeutic effects against established B16 melanoma. This therapeutic effect was mediated by CD8 T cells via perforin-mediated lysis and required the participation of type-I IFN but not IFNγ. These results suggest that similar strategies would be applicable for the design of effective vaccination for conducting clinical studies in cancer patients. [Cancer Res 2009;69(23):OF1–8]

Introduction

There is substantial evidence that the immune system and in particular CD8 T cells have the ability to recognize and in some instances eliminate tumor cells (1–3). CD8 T cells recognize tumor antigens of the surface of tumor cells as complexes formed by small peptides that associate with MHC class I molecules (4). These T-cell epitopes are derived from the processing of proteins synthesized by the tumor cells (5, 6). Numerous factors will determine whether a peptide can act as a tumor-reactive T-cell epitope and function as a tumor rejection antigen. Most importantly is whether the peptide is produced by the endogenous antigen-processing pathway and can bind with sufficient strength to MHC class I molecules to be expressed on the surface of the tumor cell to interact with CD8 T cells. In addition, peptides derived from nonmutated proteins expressed on normal tissues may not be very immunogenic due to their propensity to induce immunologic tolerance. Nevertheless, it seems that the mechanisms governing immune tolerance are not flawless, and that in many instances, autoreactive CD8 T-cell precursors can escape deletion or immune suppression and that when activated appropriately, potent autoimmune or antitumor responses can be generated. Several investigators including our group have considered using synthetic peptides representing CD8 T-cell epitopes as vaccines for treating established tumors. Numerous studies that have evaluated synthetic peptide vaccines for cancer both in humans and in animal models have yielded rather disappointing results with respect to their antitumor effects, indicating that peptides per se are not very immunogenic (7–9). This conclusion is supported by the measurements of antigen-specific CD8 T-cell responses induced by these vaccines, where in the majority of cases <1% of all CD8 T cells were shown to be antigen specific. The inadequate performance of synthetic peptide–based vaccines may be due to the use of suboptimal immune adjuvants, which in most instances fail to activate the immune system resulting in T-cell ignorance or, worse, in the induction of T-cell anergy (10, 11). The present studies were originated from the presumption that to achieve highly effective antitumor effects, peptide vaccines must be capable of generating CD8 T-cell responses of a magnitude similar to those responses observed during acute infections where 10% to 50% of all CD8 T cells are antigen specific. We present here evidence that with the appropriate combination of Toll-like receptor ligands (TLR-L) and costimulatory agonists, it is possible to achieve these high levels of CD8 T-cell responses using synthetic peptides as antigen. The intensity and duration of T-cell responses reported here have not been observed with the use of any type of subunit vaccine and can only be compared with the results obtained with adoptive T-cell therapy, which in many cases still requires subsequent vaccination and high-dose interleukin 2 (IL-2) to achieve antitumor effects (12, 13). More importantly, our results show that this vaccination strategy was highly effective in a mouse model of melanoma where the used CD8 T-cell epitope represents a peptide derived from a tissue differentiation product expressed by normal melanocytes. We believe that the results from these preclinical studies open a new venue to developing effective peptide vaccination strategies in human patients.

Materials and Methods

Mice and cell lines. Female C57BL/6 (B6), IFNγ-deficient (IFNγ−/−), and perforin-deficient (Prf−/−) mice were purchased from The Jackson Laboratory. IFNγ−/− receptor−deficient (IFNγR−/−) mice were a generous gift of Dr. Philippa Marrack (National Jewish Medical and Research Center, Denver, CO). All animal care and experiments were conducted according to our institutional animal care and use committee guidelines. The murine melanoma cell line B16F10 was provided by Dr. Jose Guevara (University of Chicago, Chicago, IL). The B16F10-Kl− cell line is a variant of B16F10 previously selected in our laboratory, which does not express H-2Kb molecules (but expresses H-2Db) even after treatment with IFNγ (using 100 ng/mL for 24 h). EL4 and its Ova-transfected variant EG7 were obtained from the American Type Culture Collection.
Results

Peptide immunization can induce large numbers of long-lasting and functional antigen-specific CD8 T cells. It has been reported that the use of agonistic mAb specific for CD40 is capable of increasing T-cell responses resulting from various types of immunizations including peptide vaccines (15–17). It is believed that anti-CD40 mAb functions as an adjuvant by preventing the induction of T-cell anergy that peptides can induce when presented to CD8 T cells by nonprofessional antigen-presenting cells (APC). However, we have observed that the CD8 T-cell responses generated in mice immunized with soluble peptide and anti-CD40 mAb exhibit clear signs of T-cell anergy since a subsequent booster peptide immunization, even in the presence of anti-CD40 mAb, was unable to elicit a significant secondary immune response. In view of this, we evaluated whether the use of TLR-Ls would improve the immune response of peptide vaccination administered with anti-CD40 mAb. As shown in Fig. 1A, the addition of a TLR-L to a vaccine consisting of peptide Ova257-264 and anti-CD40 mAb increased from 2- to 3-fold the levels of antigen-specific CD8 T cells observed in blood 6 days after the administration of the vaccine. Vaccination with peptide alone or peptide with either of these TLR-Ls without anti-CD40 mAb generated ≤0.5% tetramer-positive cells (data not shown). An identical booster immunization was applied on day 21 and the levels of antigen-specific CD8 T cells in blood were monitored every ~20 days. The data in Fig. 1B indicate that only three of the five TLR-Ls (poly-IC, GDQ, and LPS) were able to elicit secondary CD8 T-cell responses. These results indicate that peptide immunization with a vaccine containing three components,

\[ P \text{ through various time points.} \]

\[ \text{tetramer-specific CD8 T cells in peripheral blood was followed in individual mice} \]

\[ \text{the no TLR group are shown inside of each column;} \]

\[ \text{emulsified in IFA in addition with anti-CD40 mAb and poly-IC, which were} \]

\[ \text{In some instances, mice received s.c. vaccination with peptide in PBS or} \]

\[ \text{Washington, DC). LPS, GDQ, and FSL1 were purchased from InvivoGen.} \]

\[ \text{γ} \]

\[ \text{(EliSpot) assays [IFN} \]

\[ \text{evaluate using a FACSCalibur flow cytometer (BD Biosciences). For detection} \]

\[ \text{of cytokine-secreting CD8 T cells, enzyme-linked immunosorbent spot} \]

\[ \text{EliSpot] assays [IFNγ or tumor necrosis factor (TNF} \]

\[ \text{were performed as described previously (14) using purified CD8 T cells. For cytokotoxicity} \]

\[ \text{determinations, conventional 5-h chromium release assays were performed using} \]

\[ \text{freshly isolated CD8 T cells (effectors) against various target cells that were} \]

\[ \text{labeled with } 51\text{Cr in triplicate using various effectors to target ratios.} \]

\[ \text{Antitumor effects. To assess the efficacy of vaccination in the prophylactic setting, previously} \]

\[ \text{immunized mice were challenged with } 3 \times 10^5 \]

\[ \text{B16F10 cells injected through the tail vein 12 d after the last vaccination.} \]

\[ \text{To study the therapeutic effects of vaccination, mice first received an i.v.} \]

\[ \text{tumor inoculation ( } 3 \times 10^5 , 1 \times 10^5 \text{ or } 3 \times 10^5 \text{ B16F10 cells)} \]

\[ \text{and } 3 \text{ to } 7 \text{ d later (depending on the specific experiment) the mice received their} \]

\[ \text{first immunization. Antitumor effects were evaluated by the examination and} \]

\[ \text{measurements of lung tumor nodules and lung weights } \sim 4 \text{ wk after tumor} \]

\[ \text{injections (when the mice in the unvaccinated control group started to} \]

\[ \text{appear ill). In some experiments, the antitumor effects were evaluated by survival} \]

\[ \text{measurements (time to euthanasia). In all instances, groups of mice} \]

\[ \text{consisted of eight animals per group. For in vivo cell depletions [natural} \]

\[ \text{killer (NK), CD4, or CD8 T cells), mice received the following mAbs via} \]

\[ \text{i.p. injections: anti-NK (clone PK136) } 300 \text{ μg/injection, anti-CD4 (clone} \]

\[ \text{GK1.5) 300 μg/injection, and anti-CD8 (clone 2.43) 500 μg/injection. Depletions} \]

\[ \text{were confirmed by analysis of blood samples using flow cytometry} \]

\[ \text{(data not shown). In some instances, survivor mice were rechallenged s.c. in} \]

\[ \text{the posterior flank with either B16F10 or B16F10-Kb− cells (5 } \times 10^5 \text{ mouse).} \]

\[ \text{Tumor growth was monitored every 2 to 4 d in individually tagged mice by} \]

\[ \text{measuring two opposing diameters with a set of callipers. Results are} \]

\[ \text{presented as the mean tumor size (area in mm} \]

\[ \text{were performed as two-way ANOVA test} \]

\[ \text{(*, } P < 0.0001 \text{ for TriVax versus peptide-anti-CD40 mAb).} \]

Peptides, antibodies, and tetramers. Synthetic peptides representing H-2Kb-restricted CD8 T-cell epitopes, Trp2180-188 (SVYDFFVWL), Ova257-264 (SIINFEKL), and Ova255-64 (KVFDRKL) were purchased from A&A Labs. Rat anti-mouse CD40 monoclonal antibody (mAb; anti-CD40 mAb) was prepared from the FGK45.5 hybridoma. Phycoerythrin-conjugated H-2Kb/Ova257-264 tetramer was purchased from Beckman Coulter. H-2Kb/Trp2180-188 tetramer was provided by the NIAID Tetramer Facility.

Immunizations. For TriVax immunizations, mice were immunized i.v. (unless otherwise noted) with a mixture of 200 μg peptide, 50 μg anti-CD40 mAb, and a TLR-L (50 μg poly-IC, 100 μg CpG-1826, 30 μg lipopolysaccharide (LPS), 100 μg Gardiquimod (GDQ), or 25 μg FSL1). CpG-1826 was prepared by the Mayo Clinic Molecular Core Facility. Poly-IC (Hiltonol, a clinical grade stabilized formulation containing poly-γ-L-lysine and carboxymethyl cellulose) was provided by Dr. Andres Salazar (Oncovir Inc., Washington, DC). LPS, GDQ, and FSL1 were purchased from InvivoGen. In some instances, mice received s.c. vaccination with peptide in PBS or emulsified in IFA in addition with anti-CD40 mAb and poly-IC, which were also injected s.c. in close proximity to the peptide.

Evaluation of cellular immune responses. For tetramer staining, either blood or splenocytes were stained with FITC-conjugated MHC class II, PerCP Cy5.5-conjugated CD8α (both from eBioscience), and phycoerythrin-conjugated Kb/peptide tetramers for 40 min in ice. Fluorescence was evaluated using a FACScalibur flow cytometer (BD Biosciences). For detection of cytokine-secreting CD8 T cells, enzyme-linked immunosorbent spot (EliSpot) assays [IFNγ or tumor necrosis factor (TNFα)] were performed as
synthetic peptide, anti-CD40 mAb and poly-IC, from hereafter called TriVax, generates antigen-specific CD8 T-cells that are capable of dramatically expanding upon a booster immunization.

**Evaluation of TriVax using a peptide representing a CD8 tumor epitope.** Next, we evaluated TriVax using a CD8 peptide epitope derived from the melanosomal tyrosinase-related protein 2 (Trp2, also known as dopachrome tautomerase). Trp2180-188 functions as an H-2Kb-restricted CD8 T-cell epitope expressed by B16 melanoma (18, 19). Trp2180-188-TriVax immunization induced a strong primary CD8 T-cell response that could be increased by ~3-fold with a subsequent TriVax boost (Fig. 2A). The functional activity of CD8 T cells isolated from spleens was shown in EliSpot assays (Fig. 2B). These responses were evident not only against peptide-pulsed APC but also toward B16 melanoma cells. freshly isolated spleen CD8 T cells (without further peptide restimulation) displayed high levels of cytotoxic activity (Fig. 2C). The levels of antigen-specific CD8 T cells in blood could be further increased (to 40–60% tetramer positive) when the mice received additional immunizations, remaining high for several months and presumably were responsible for the generation of extensive vitiligo (Supplementary Fig. S1).

Next, we assessed whether the CD8 T cells generated with TriVax immunization would be capable of displaying *in vivo* antitumor effects. Mice received various modes of TriVax, and 12 days after the last immunization they were challenged with B16 cells. We also compared the antitumor efficacy of a single immunization versus a prime/boost. The results indicate that TriVax with Trp2180-188 offered significant protection against B16 and that the prime/boost protocol was more effective than a single immunization (Fig. 2D).

**Therapeutic effects of TriVax against established B16 melanoma.** Next, we studied whether TriVax would offer a therapeutic benefit against established B16 tumors. Mice received an i.v. injection of B16F10 cells, and 3 days later, the vaccinations were initiated. On day 24, when the control mice appeared sick, all mice were euthanatized and their lungs were evaluated for the presence of tumors. As shown in Fig. 3A, the lungs from mice that received Trp2180-188-TriVax had little evidence of disease compared with the
control mice. Furthermore, the mice that received the Trp2180-188-TriVax had high levels of antigen-specific CD8 T cells, whereas the mice that received the Ova 55-63-TriVax did not have any detectable Trp2180-188-specific CD8 T cells (Fig. 3B and C). We also evaluated the therapeutic effects of TriVax against established (7 day) s.c. B16 tumors. Although no tumor rejections were obtained in this more challenging model, a significant reduction in the tumor growth rate was observed (Supplementary Fig. S2).

The antitumor effects of TriVax were evaluated against more established tumors. Significant antitumor effects were still observed when mice received their first immunization either 5 or 7 days after the i.v. tumor inoculation (Supplementary Fig. S2). However, the lungs from the Trp2180-188-TriVax mice on day 7 had some evidence of small tumor nodules, although these mice exhibited large numbers of antigen-specific CD8 T cells (Supplementary Fig. S3).

Vaccination with peptides can induce T-cell anergy, especially when the peptides are administered in soluble form via an i.v. route. To avoid this problem, peptides are administered via a s.c. route, formulated in a water-in-oil emulsions such as incomplete Freund's adjuvant (IFA). Thus, we compared the therapeutic antitumor responses of the conventional TriVax i.v. immunization with a TriVax modification where the peptide was injected s.c. in IFA and where the poly-IC and anti-CD40 mAb were also injected in close proximity to the peptide emulsion. The s.c. administration of Trp2180-188/IFA-TriVax exhibited some therapeutic benefit but was not as effective as the i.v. Trp2180-188-TriVax (Fig. 4A). These results may be explained by the differences of the Trp2180-188-specific CD8 T-cell responses observed with these vaccines (Fig. 4B and C). Suboptimal antitumor effects were also obtained when TriVax formulated with soluble peptide (no IFA) was administered via a s.c. route compared with the i.v. route (Supplementary Fig. S4), indicating that systemic administration of the vaccine was important to generate a therapeutic effect.

TriVax was evaluated for its survival benefit of in the pulmonary B16 tumor model. The results shown in Fig. 5A show that with a high-tumor dose, TriVax increased the medium survival by ∼25 days, but by day 58, all the mice perished. However, the survival advantage of TriVax was enhanced when the tumor dose was decreased. Interestingly, postmortem examination of the Trp2180-188-TriVax-immunized mice that died in these experiments revealed
only few, if any, lung tumor nodules (Supplementary Fig. S5). However, in some instances, tumors developed in the peritoneal cavity or in the pericardium (data not shown). To assess the presence of long-term immunity, the surviving TriVax-immunized mice were rechallenged with a s.c. B16 injection. Half of the mice received B16F10 cells and the other half were challenged with a B16F10 tumor variant that does not express the H-2Kb molecule (B16F10-Kb−). None of the immune mice developed tumors when rechallenged s.c. with B16F10 cells (Fig. 5B). On the other hand, the mice challenged with the B16F10-Kb− cells developed tumors but the growth rate was significantly slower than the control group that was challenged with the same tumor, suggesting that these mice may have acquired additional antitumor immune mechanisms (antibodies, CD4 T-cell responses, or H-2Db–restricted CD8 T-cell epitope) capable of decreasing growth of tumors not expressing the H-2Kb–restricted Trp2180-188 CD8 epitope.

**Evaluation of the immune effector mechanisms of TriVax.**
Lastly, we assessed the roles of various immune effector cells such as NK and CD4 T cells that could participate in the induction of the CD8 T-cell response and/or synergize with CD8 T cells in fighting the established tumors. Depletion of either CD4 T cells or NK cells had no significant deleterious effect in the survival advantage provided by TriVax, whereas depletion of CD8 T cells completely abolished the antitumor effects (Fig. 6A). CD8 T cells can exert their antitumor effect via numerous effector mechanisms such as perforin-mediated cytolysis (20) and IFNγ (21–23). In addition, type-I IFN has been implicated as being critical in CD8 T-cell responses (24). Thus, the efficacy of Trp2180-188-TriVax was evaluated.
in mice deficient for perforin, IFNγ, or type-I IFN receptor. The therapeutic benefit of TriVax was absent in mice that lack perforin or type-I IFN receptor, indicating that these molecules are crucial for the rejection of established tumors (Fig. 6B). Conversely, an increase in the therapeutic effect of Trp2180-188-TriVax was observed in mice deficient for IFNγ, suggesting that this cytokine may somehow inhibit the function of CD8 T cells in this model. Cell-mediated cytotoxicity assays revealed that the IFNαβR−/− mice had decreased CD8 T-cell activity compared with B6 wild-type (WT) mice, whereas the IFNγ-deficient mice had slightly higher levels of activity (Fig. 6C). A similar pattern was observed when CD8 T cells in these mice were evaluated by tetramer analysis (Supplementary Fig. S6). Overall, these results show that the anti-tumor effect of Trp2180-188-TriVax in this tumor model is mostly derived from perforin-mediated lysis of tumor cells by CD8 T cells.

Discussion

The development of T cell–based immunotherapy for cancer continues to be a major challenge and one of the top priorities for tumor immunologists. The most clear and dramatic example showing that T lymphocytes can eliminate large tumors come from studies using adoptive T-cell therapy (12, 25). Adoptive T-cell therapy usually requires the administration of high doses of IL-2 and, in many instances, lymphoablation and a subsequent strong immunization (e.g., recombinant vaccinia) to achieve antitumor effects (13). Under these conditions, very high numbers of antigen-specific T cells are achieved, which rarely can be observed with any conventional vaccines without the use of adoptive T-cell transfers. Numerous homeostatic mechanisms operate in the immune system to ensure that the number of T cells for various antigenic specificities increase (expansion phase) when required to combat...
disease and later decrease (contraction phase) when the threat subsides and are no longer needed (26). To successfully combat infections, the immune system overrides homeostasis, allowing huge expansion of pathogen-specific T lymphocytes (27). The majority of the peptide-based vaccines fail to generate the vast CD8 T-cell responses obtained by adoptive T-cell therapy/lymphoablation or observed during acute infections, and therefore, it is not surprising that these perform poorly against tumors.

We present here the results of our attempts to design and implement a peptide vaccination strategy that mimics an acute infection generating CD8 T-cell responses of the magnitude observed in such infections and achieved by adoptive T-cell therapy. We assessed the adjuvant activity of five compounds known to stimulate different TLR family members. After primary immunization, all of the TLR-L tested significantly increased the levels of antigen-specific CD8 T cells compared with the use of peptide and anti-CD40 mAb alone (Fig. 1A). Interestingly, only some of the TLR-L were able to generate secondary immune responses (Fig. 1B). The results obtained with poly-IC were most dramatic, where at the peak of the response (6 days postboost), nearly 80% of the CD8 T cells in blood were antigen specific. To our knowledge, this could be the first example where these high levels of CD8 T cells are generated using any kind of noninfectious vaccine and without a prior infusion of TCR transgenic T cells. At present, we do not know the reasons why the various TLR-L performed so differently as adjuvants in the TriVax immunization strategy. These differences could be due to variations in stability, biodistribution, and pharmacokinetics of these compounds or due to their capacity to stimulate different cells of the immune system. In addition, poly-IC stimulates the immune system through other pathways in addition to TLR3 (28–30). The combination of TLR-L, anti-CD40 mAb, and peptide for the generation of CD8 T-cell responses has been used by other groups, but for unknown reasons, their results differ substantially from ours. Ahonen and colleagues (17) first reported the generation of strong CD8 T-cell responses to Ova257-263, but subsequent boosters were not effective in generating secondary immune responses. The same group was unable to elicit CD8 T-cell responses to the Trp2180-188 natural epitope (SVYDFFVWL) unless they used an analogue synthetic peptide (31). Moreover, even when the analogue peptide was used, booster

![Figure 6](https://cancerres.aacrjournals.org/doi/10.1158/0008-5472.CAN-09-2019)
The antitumor therapeutic effect of TriVax against B16 melanoma ob-
but could also be detrimental for tumor eradication. The increased T cells or NK cells (Fig. 6). In addition, our results indicate that tu-
tude of the antigen-specific T-cell response is important.

B16 melanoma, to achieve effective antitumor responses, magni-
results, we believe that the capacity of a vaccine to induce CD8 T

antibody during the initiation of these studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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