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Maximizing CD8⁺ T cell Responses Elicited by Peptide Vaccines Containing CpG Oligodeoxynucleotides

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Abstract

We assessed the ability of several factors to increase the size of tumor-antigen-specific CD8⁺ T cell responses elicited by vaccines incorporating peptides and CpG-containing oligodeoxynucleotides (CpG). Neither granulocyte-macrophage colony-stimulating factor (GM-CSF) nor an immunogenic MHC class II-presented “helper” peptide increased the size of epitope-specific CD8⁺ T cell responses elicited by peptide+CpG-containing vaccines. In contrast, low-dose subcutaneous interleukin (IL)-2 dramatically increased the size of splenic and peripheral blood epitope-specific CD8⁺ T cell responses generated by peptide+CpG-containing vaccines. Moreover, peptide+CpG-containing vaccines plus low-dose IL-2 mediated anti-tumor immunity. A prime-boost vaccination schedule elicited larger CD8⁺ T cell responses than a weekly vaccination schedule. Including larger doses of peptide in vaccines led to larger vaccine-elicited CD8⁺ T cell responses. Clinical trials of CpG-containing peptide vaccines are ongoing. These findings suggest strategies to increase the size of CD8⁺ T cell responses generated by CpG-containing peptide vaccines that could be tested in future clinical trials.

Keywords

vaccination; tumor immunity; peptides; cytokines; T cells

Introduction

T cells can cause objective remissions of melanoma in humans [1], and CD8⁺ T cells specific for tumor-associated antigens can inhibit growth of poorly immunogenic tumors in mice [2; 3;4;5]. Anti-cancer vaccines inconsistently cause objective clinical anti-tumor responses [6], and anti-cancer vaccination strategies that have been tested in clinical trials generally produce median CD8⁺ T cell responses in which less than 1% of CD8⁺ T cells are specific for a particular epitope [7;8;9]. Development of vaccination regimens capable of eliciting CD8⁺ T cell responses of substantially greater magnitude than those elicited by current vaccines is important because the anti-tumor efficacy of vaccines has been shown to be correlated with the size of vaccine-elicited tumor-antigen-specific CD8⁺ T cell responses [10].

Oligodeoxynucleotides that contain unmethylated CpG motifs (CpG) potentially enhance T cell responses in multiple murine vaccination models [3;4;11;12;13]. Vaccines consisting of CpG and immunogenic peptides emulsified in incomplete Freund's adjuvant (IFA) are currently

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being tested in clinical trials. These vaccines have elicited some of the strongest CD8⁺ T cell responses ever generated by vaccines in humans [14]. Although interleukin (IL)-2 has been only modestly effective as a single-agent vaccine adjuvant in mice [2;15;16] and IL-2 has not been effective at enhancing CD8⁺ T cell responses elicited by peptide vaccines in humans [17], we have recently shown that IL-2 and CpG synergize to dramatically increase the magnitude of CD8⁺ T cell responses elicited by peptide vaccines [4;18]. The dose of IL-2 administered is a critical factor due to the significant toxicity of this cytokine [19]. For clinical trial design, it is important to determine whether low-dose subcutaneously administered (sc) IL-2 can enhance CD8⁺ T cell responses generated by peptide+CpG-containing vaccines.

We hypothesized that adjuvants other than IL-2 might also enhance the effectiveness of CpG-containing vaccines. Granulocyte-macrophage colony stimulating factor (GM-CSF) has enhanced vaccination in murine models [5;20;21]. In contrast, GM-CSF has not consistently increased CD8⁺ T cell responses in clinical trials of peptide vaccination [9;22]. Another strategy that can enhance CD8⁺ T cell responses in mice is to include a peptide that is capable of generating a CD4⁺ “helper” T cell response in a vaccine that also contains an immunogenic peptide presented by MHC class I [23].

Because CpG-containing peptide vaccination regimens are currently undergoing clinical trials in cancer patients, it is important to optimize these regimens for generation of the largest possible T cell responses. We evaluated the abilities of an immunogenic MHC class II-presented “helper” peptide, GM-CSF, and low dose IL-2 to increase CD8⁺ T cell responses generated by CpG-containing peptide vaccines. We found that GM-CSF and immunogenic MHC class II-presented peptides did not increase CD8⁺ T cell responses generated by CpG-containing vaccines. Low-dose sc IL-2 dramatically increased the magnitude of CD8⁺ T cell responses generated by CpG-containing vaccines. In addition, the schedule of vaccine administration and the dose of peptide included in vaccines were important factors in optimizing CpG-containing peptide vaccination. Finally, we demonstrated that peptide+CpG-containing vaccines combined with low-dose sc IL-2 can mediate epitope-specific anti-tumor immunity.

Methods and Materials

Mice and vaccine ingredients

C57BL/6 mice housed under pathogen-free conditions were used in all experiments. All animal studies were approved by the National Cancer Institute Center for Cancer Research Animal Care and Use Committee. The B16F1 melanoma cell line was purchased from ATCC.

Amino acids 180–188 of the TRP-2 protein form an immunogenic MHC class I-restricted epitope that is presented by H-2 K^b [3;5;24]. TRP-2 is a non-mutated protein that is expressed by normal melanocytes in C57BL/6 mice [24]; therefore, it is subject to self-tolerance. Amino acids 257–264 of the ovalbumin protein (OVA_{257–264}) form an immunogenic epitope that is presented by H-2 K^b [11]. Amino acids 366–374 of the influenza A nucleoprotein (NP_{366–374}) [2] and amino acids 49–57 of the human papillomavirus E7 protein (E7_{49–57}) [25] form immunogenic epitopes presented by H-2 D^b. Amino acids 128–140 of the hepatitis B core protein (HBC_{128–140}) form an epitope presented by H-2 I-A^b that can elicit CD4⁺ T cell responses [23].

All peptides were synthesized and purified to 95% purity by Biopeptide Co. LLC. Murine GM-CSF was purchased from Peprotech and dissolved in phosphate-buffered saline. Human recombinant IL-2 was obtained from the National Cancer Institute Biological Resources Branch. The CpG used in our experiments, CpG 1826 [26], was purchased from Coley Pharmaceutical Group. Incomplete Freund's Adjuvant (IFA) was purchased from Sigma.

Vaccine preparation

In the experiments presented in Figure 1, complete priming vaccines consisted of the following components in IFA: 50 µg CpG, 50 µg TRP-2_{180–188}, and 60 µg HBC_{128–140}. Complete boost vaccines were prepared in an identical manner as priming vaccines except 100 µg of TRP-2_{180–188} and 120 µg of HBC_{128–140} were included. When GM-CSF was administered, 4 µg were injected subcutaneously (sc) at the vaccination site 24 hours and 8 hours prior to each priming and boost vaccination. In some experiments, certain components were eliminated from the vaccines. When HBC_{128–140} was eliminated it was replaced with an equal volume of DMSO. When CpG was eliminated it was replaced with an equal volume of tris-EDTA buffer. When GM-CSF was eliminated it was replaced by phosphate-buffered-saline injections.

In the experiments described in Figure 2-5, low-dose CpG vaccines were administered that contained 50 µg of TRP-2_{180–188} or OVA_{257–264} and 10 µg CpG in IFA. When IL-2 was administered, either 40,000 IU were administered intraperitoneally (ip) twice each day (high-dose IL-2) or 30,000 IU were administered sc once each day (low-dose IL-2). When IL-2 was not administered, control buffer, containing human serum albumin and mannitol in PBS, was injected in place of IL-2.

Vaccine injections were administered on various schedules detailed in the results section. Regardless of the schedule used, the first and third vaccines were given sc at the base of the tail; the second vaccination was administered sc on the left side. If a boost vaccination was given, it was injected at the base of the tail sc.

Antibodies

These antibodies from BD Pharmingen were used: anti-CD3ε (clone 145–2C11), anti-IFNγ (clone XMG1.2), anti-CD28 (clone 37.51), anti-CD16/CD32 (clone 2.4G2), and anti-CD62L (clone MEL-14). Anti-CD127 (clone A7R34) from eBioscience was used. Anti-CD8α (clone CT-CD8a) from Caltag was used.

Peptide stimulation followed by intracellular cytokine staining (ICCS)

The percentage of CD8⁺ T cells specific for TRP-2_{180–188} was determined by stimulating splenocytes with peptides followed by intracellular cytokine staining (ICCS). Mice were sacrificed. Splenocytes were RBC depleted and suspended at 3.5×10^6 live cells per ml. For each mouse, two tubes were prepared. Each tube contained 3.5×10^6 splenocytes, 1 µl Golgi Plug (Pharmingen), and 2 µg/ml of a stimulatory anti-CD28 antibody (anti-CD28 was used only in the experiments reported in Figures 1 and 2A-C). For each mouse, 20 µg/ml TRP-2_{180–188} was added to one tube and 20 µg/ml of the negative control peptide OVA_{257–264} was added to the other tube. All tubes were incubated at 37° for 6 hours. The cells were then washed and surface stained for CD3 and CD8. The cells were permeabilized and stained for intracellular IFNγ according to the instructions of the Cytofix/Cytoperm kit (Pharmingen). Analysis was performed with Cellquest™ software (Becton Dickinson). For each mouse, a tube containing cells stimulated with TRP-2_{180–188} and another tube containing cells stimulated with OVA_{257–264} were analyzed. The percentage of CD8⁺ T cells specific for TRP-2_{180–188} was calculated as the percentage of CD3⁺CD8⁺IFNγ⁺ events with TRP-2_{180–188} stimulation minus the percentage of CD3⁺CD8⁺IFNγ⁺ events with OVA_{257–264} stimulation.

Peripheral blood ICCS assay

Peripheral blood was collected and RBC-depleted by two rounds of lysis with ACK lysing buffer (BioWhittaker). ICCS assays were performed as described above for ICCS assays

carried out with splenocytes except that 1×10^6 live peripheral blood WBC were used in stimulation cultures.

Quantitation of the absolute number of splenic CD8⁺ T cells specific for TRP-2₁₈₀₋₁₈₈

The absolute number of live splenocytes that expressed CD3 and CD8 was determined by flow cytometry as described previously [4]. The absolute number of splenic TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cells was determined by multiplying the absolute number of CD3⁺CD8⁺ splenocytes by the percentage of CD8⁺ T cells specific for TRP-2₁₈₀₋₁₈₈ determined during the ICCS assay.

Tetramer staining

Peripheral blood was collected and depleted of RBCs by two rounds of lysis with ACK lysing buffer. Live white blood cells (WBC) were counted using trypan blue for dead cell exclusion. The number of WBC per μ l of blood was calculated for each mouse. To block nonspecific binding of tetramer to CD8, WBC were stained for fifteen minutes at room temperature with anti-CD8. Next, the cells were stained with OVA₂₅₇₋₂₆₄-K^b tetramers (Coulter), anti-CD62L, and anti-CD127 for 25 minutes at room temperature. The cells were then washed and flow cytometry acquisition was performed immediately. Flow cytometry data analysis was performed with Cellquest™ software. Two control-vaccinated mice that were vaccinated with either NP₃₆₆₋₃₇₄ or E7₄₉₋₅₇ were included in each experiment to control for nonspecific tetramer binding. For each OVA₂₅₇₋₂₆₄-vaccinated mouse, the percentage of CD8⁺ lymphocytes that specifically bound OVA₂₅₇₋₂₆₄-K^b tetramers was calculated by subtracting the mean percentage of CD8⁺ lymphocytes from the control vaccinated mice in a given experiment that bound OVA₂₅₇₋₂₆₄-K^b tetramers from the percentage of CD8⁺ lymphocytes from each OVA₂₅₇₋₂₆₄-vaccinated mouse that bound OVA₂₅₇₋₂₆₄-K^b tetramers. For each vaccinated mouse, the absolute number of OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood was calculated. First, the absolute number of CD8⁺ lymphocytes per μ l of blood was calculated by multiplying the number of WBC per μ l of blood by the percentage of WBC that were CD8⁺ lymphocytes. The absolute number of CD8⁺ lymphocytes per μ l of blood was multiplied by the percentage of CD8⁺ lymphocytes that bound OVA₂₅₇₋₂₆₄-K^b tetramers to determine the absolute number of OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood.

Tumor Therapy Experiments

B16F1 cells were rapidly proliferating with a viability of greater than 95% at the time of injection. The cells were kept on ice and mixed well prior to each injection. Mice were injected with 30,000 tumor cells sc on the right side. For each tumor therapy experiment, mice were injected with tumor cells and then randomly distributed to different treatment or control groups. Tumor size was measured with calipers every three days starting 10 days after tumor injection. The longest length and the length perpendicular to the longest length were multiplied to obtain the tumor size (area) in mm². When the tumor size reached 200 mm² or ulceration developed, the mice were sacrificed.

Statistical analysis

Groups were compared using the two-tailed Mann-Whitney test. In cases where three or four groups were compared (Figures 1F and 5A), $P < 0.01$ should be considered statistically significant in accordance with the Bonferroni correction. In all other cases, $P < 0.05$ should be considered statistically significant. Survival curves were compared using the log-rank test. In all graphs, the mean and the standard error of the mean are shown. 2–3 experiments of each type were conducted and the results of all experiments were combined for graphical presentation and statistical analysis.

Results

Adjuvants dramatically increase the magnitude of CD8⁺ T cell responses elicited by peptide vaccines.

A vaccination regimen of TRP-2_{180–188} in IFA administered weekly for four doses elicited a mean TRP-2_{180–188}-specific response of 0.06% of CD8⁺ T cells (Figure 1A). We hypothesized that the magnitude of the CD8⁺ T cell response elicited by simple TRP-2_{180–188} in IFA vaccines could be increased by adding additional adjuvants such as CpG, GMCSF, and HBC_{128–140}. We also hypothesized that a prime-boost vaccination schedule consisting of a series of priming vaccinations with a short time interval between vaccinations followed by a boost vaccination would elicit larger CD8⁺ T cell responses than a weekly vaccination schedule. To test these hypotheses, we administered a regimen in which vaccinations consisted of TRP-2_{180–188}, HBC_{128–140}, and CpG in IFA. All vaccinations were preceded by GM-CSF injections at the vaccine site. When we administered this vaccination regimen to mice, strong CD8⁺ T cell responses against TRP-2_{180–188} but minimal responses against the negative control peptide OVA_{257–264} were detected by ICCS assay (Figures 1B and 1C).

The magnitude of CD8⁺ T cell responses elicited by peptide vaccination depends on the vaccination schedule.

A prime-boost schedule that consisted of a series of priming vaccines on days 0, 3, and 6 and a boost vaccine on day 21 elicited larger TRP-2_{180–188}-specific CD8⁺ T cell responses than a regimen in which vaccines were administered on a weekly schedule (Figure 1D).

The magnitude of CD8⁺ T cell responses elicited by peptide vaccination depends on the peptide dose.

We hypothesized that the magnitude of CD8⁺ T cell responses elicited by peptide vaccines would increase when higher doses of peptide were included in the vaccines. When we compared the TRP-2_{180–188}-specific CD8⁺ T cell responses elicited by vaccines containing doses of TRP-2_{180–188} that differed by a factor of ten, the mice receiving the higher dose of TRP-2_{180–188} had larger TRP-2_{180–188}-specific CD8⁺ T cell responses than the mice receiving the lower dose of TRP-2_{180–188} (Figure 1E).

CpG was the critical adjuvant in the complete vaccination regimen.

We administered the complete vaccination regimen of TRP-2_{180–188}+CpG+IFA +HBC_{128–140} along with GM-CSF or vaccination regimens with one component at a time omitted. We measured the resulting TRP-2_{180–188}-specific CD8⁺ T cell responses by ICCS assay. CpG was the critical adjuvant in the complete vaccination regimen because only omission of this adjuvant caused a statistically significant decrease in TRP-2_{180–188}-specific CD8⁺ T cell responses compared to the responses elicited by the complete regimen (Figure 1F). Omission of GM-CSF or HBC_{128–140} from CpG-containing vaccination regimens did not decrease vaccine-elicited CD8⁺ T cell responses.

Low doses of CpG and IL-2 synergize to increase TRP-2_{180–188}-specific CD8⁺ T cell responses.

Addition of low-dose sc IL-2 to CpG-containing peptide vaccines dramatically increased vaccine-elicited TRP-2_{180–188}-specific CD8⁺ T cell responses (Figure 2A–2C). Larger TRP-2_{180–188}-specific CD8⁺ T cell responses were generated when high-dose ip IL-2 was administered after TRP-2_{180–188}+CpG-containing vaccines than when low-dose sc IL-2 was administered after identical vaccines (Figure 2D–2E).

IL-2 increases peripheral blood CD8⁺ T cell responses generated by peptide+CpG-containing vaccines.

In human clinical trials, vaccine-elicited T cell responses are usually measured in the peripheral blood; therefore, we carried out an extensive analysis of peripheral blood CD8⁺ T cell responses generated by peptide+CpG+IFA vaccines administered with or without IL-2.

OVA₂₅₇₋₂₆₄+CpG+IFA vaccines generated more OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ peripheral blood lymphocytes when low-dose sc IL-2 was combined with the vaccinations (Figures 3A and 3C). In these experiments, OVA₂₅₇₋₂₆₄+CpG+IFA vaccination alone generated a median of 1.0 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocyte per μ l of blood while the same vaccination regimen combined with low dose sc IL-2 generated a median of 8.5 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood. Addition of high-dose ip IL-2 to OVA₂₅₇₋₂₆₄+CpG+ IFA vaccines was more effective than addition of low-dose sc IL-2 to the same vaccination regimen at increasing the number of OVA₂₅₇₋₂₆₄-K^b tetramer-binding peripheral blood CD8⁺ lymphocytes (Figures 3B and 3D). In a series of experiments that tested the impact of high-dose IL-2 on vaccine-elicited CD8⁺ T cell responses, OVA₂₅₇₋₂₆₄+CpG+IFA vaccines combined with high-dose ip IL-2 generated a median of 19.9 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood while the same vaccination regimen without IL-2 generated a median of 0.4 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood. All of the OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell responses described above were measured seven days after the final vaccination of a priming series of three vaccinations. When mice were vaccinated on days 0, 3, and 6 with OVA₂₅₇₋₂₆₄+CpG+IFA vaccines and responses were measured twenty-eight days after the final vaccination, mice that received vaccinations plus low-dose sc IL-2 had a median of 0.4 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood and mice that received OVA₂₅₇₋₂₆₄+CpG+IFA vaccines without IL-2 had a median of 0.3 OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes per μ l of blood (n=8 mice per group).

CD8⁺ T cells can be divided into different classes based on expression of CD62L and CD127 [27]. In mice that received OVA₂₅₇₋₂₆₄+CpG+IFA vaccines plus systemic IL-2, OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes were predominately CD62L-negative, CD127-negative effector T cells regardless of whether the mice received low-dose or high-dose IL-2 (Figure 3E). Similarly, in mice that were vaccinated with OVA₂₅₇₋₂₆₄+CpG+IFA vaccines but did not receive IL-2, the mean percentage of OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes that were the CD62L-negative, CD127-negative effector T cells was 72%.

Functional peripheral blood TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses elicited by TRP-2₁₈₀₋₁₈₈+CpG+IFA vaccines were increased by low-dose sc IL-2 when responses were measured as a percentage of CD8⁺ T cells (Figures 4A and 4B) or as an absolute number (Figure 4C).

TRP-2₁₈₀₋₁₈₈+CpG-containing vaccines combined with low-dose sc IL-2 can inhibit growth of the poorly immunogenic B16F1 melanoma.

In order to assess the anti-tumor efficacy of peptide+CpG vaccines combined with IL-2, we carried out a series of experiments using the B16F1 melanoma. B16 is poorly immunogenic. Because B16F1 expresses very low levels of major histocompatibility complex class I molecules, it is a difficult test for a vaccine that elicits CD8⁺ T cell responses [28;29]. B16F1 expresses the TRP-2 protein [24]. We injected mice with B16F1, and later the same day we initiated vaccination with either TRP-2₁₈₀₋₁₈₈+CpG in IFA vaccines or the negative control peptide E7₄₉₋₅₇ and CpG in IFA. Following completion of the vaccinations, both groups received low-dose IL-2. Tumor growth was inhibited in mice that received TRP-2₁₈₀₋₁₈₈-containing vaccines relative to mice that received E7₄₉₋₅₇-containing vaccines (Figure 5A). Moreover, the mice that received TRP-2₁₈₀₋₁₈₈-containing vaccines had increased survival

relative to the E7_{49–57}-vaccinated mice (Figure 5B). Because both groups of mice were treated identically except for the peptide included in their vaccines, the tumor growth inhibition and increased survival in TRP-2_{180–188}-vaccinated mice relative to E7_{49–57}-vaccinated mice is epitope-specific and not due to non-epitope-specific effects of CpG or IL-2. B16F1 tumor growth in mice that were vaccinated with TRP-2_{180–188}+CpG+IFA vaccines combined with low-dose sc IL-2 was compared to B16F1 tumor growth in mice that were vaccinated in an identical manner and treated with high-dose ip IL-2. There was not a significant difference in B16F1 tumor growth rates when the mice that received low-dose sc IL-2 were compared to the mice that received high-dose ip IL-2 (Figure 5A).

Discussion

CpG-containing peptide vaccines are promising anti-cancer therapies because they elicit large tumor-antigen-specific CD8⁺ T cell responses in humans [14]. Clinical trials [7;8;9] and the results presented in Figure 1A demonstrate that peptide in IFA vaccines elicit small epitope-specific CD8⁺ T cell responses. When we added CpG, GM-CSF, and HBC_{128–140} to TRP-2_{180–188} in IFA vaccines, a dramatic increase in TRP-2_{180–188}-specific CD8⁺ T cell responses occurred. In addition, a prime-boost schedule in which three priming vaccinations were administered with only three days between vaccinations followed by a boost vaccination fifteen days later elicited larger TRP-2_{180–188}-specific CD8⁺ T cell responses than a schedule in which identical vaccines were administered on a weekly schedule (Figure 1D). The short time interval between priming vaccinations might supply sustained toll-like-ligand receptor signaling, allowing vaccines to break tolerance and elicit large CD8⁺ T cell responses [30].

While HBC_{128–140} has been shown to enhance generation of CD8⁺ T cell responses by peptide vaccines also containing an MHC class-I-presented peptide [23], addition of HBC_{128–140} to TRP-2_{180–188}+CpG-containing vaccination regimens did not increase TRP-2_{180–188}-specific CD8⁺ T cell responses. This is possibly due to overlap in the function of CpG and CD4⁺ helper T cells. Both CpG [12] and CD4⁺ helper T cells [31] probably enhance vaccination in part by inducing DC maturation.

GM-CSF did not increase the size of vaccine-elicited TRP-2_{180–188}-specific CD8⁺ T cell responses elicited by peptide+CpG+IFA vaccines (Figure 1F). This is somewhat surprising because transduction with the gene for GM-CSF enhanced the anti-tumor efficacy of tumor cell vaccines [5;20], and CpG enhanced the anti-tumor efficacy of a GM-CSF-transduced tumor cell vaccine [32]. Cytotoxic T lymphocyte induction by a multiepitope peptide vaccine was increased by GM-CSF [21]. In contrast, clinical trials showed no to minimal increases in peptide vaccine-elicited CD8⁺ T cell responses by GM-CSF [9;22]. Despite the ability of GM-CSF to enhance certain types of vaccination, GM-CSF can also inhibit vaccine-elicited CD8⁺ T cell responses via induction of myeloid suppressor cells [33].

In contrast to GM-CSF, low-dose sc IL-2 dramatically increased vaccine-elicited CD8⁺ T cell responses (Figure 2A–2C, Figure 3, Figure 4). These findings add to our previous findings of synergism between CpG and IL-2 in vaccine regimens that incorporated higher doses of both IL-2 and CpG [4] by demonstrating that low dose sc IL-2 can increase CD8⁺ T cell responses elicited by peptide+CpG-containing vaccines. Our finding of an enhancement of CpG-containing vaccine responses by IL-2 is consistent with the work of others which demonstrated an increase in anti-viral CD8⁺ T cell responses when low-dose IL-2 was administered during the contraction phase of T cell responses [34]. Toxicity was not evident in mice that received either low-dose sc IL-2 or higher-dose IL-2 combined with CpG-containing peptide vaccines.

We found that TRP-2_{180–188}-specific CD8⁺ T cell responses elicited by peptide+CpG-containing vaccines were larger when a larger dose of peptide was included in the vaccines

(Figure 1E). This finding is consistent with work that demonstrated an increase of CD8⁺ T cell responses with increasing antigen dose in a viral vaccination model [35]. Importantly, the avidity of CD8⁺ T cells elicited by vaccines containing a low dose of peptide was equivalent to the avidity of CD8⁺ T cells elicited by vaccines containing a ten-fold higher dose of peptide (our unpublished data).

One goal of this work was to answer questions that are important for the design of clinical trials. Because vaccine-elicited T cell responses are generally measured in the peripheral blood of patients enrolled on clinical trials, we performed an extensive series of experiments that measured vaccine-elicited CD8⁺ T cell responses in the blood of vaccinated mice. Addition of low-dose sc IL-2 to OVA₂₅₇₋₂₆₄+CpG+IFA vaccines caused an 8.5-fold increase in the median absolute number of vaccine-elicited OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ peripheral blood lymphocytes (Figure 3C). Addition of high-dose ip IL-2 to OVA₂₅₇₋₂₆₄+CpG+IFA vaccines caused a 49.8-fold increase in the median absolute number of vaccine-elicited OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ peripheral blood lymphocytes (Figure 3D). The greater increase in vaccine-elicited CD8⁺ T cell responses caused by high-dose ip IL-2 compared to low-dose sc IL-2 in these experiments is consistent with our experiments that demonstrated larger vaccine-elicited splenic TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses with administration of high-dose ip IL-2 than with administration of low-dose sc IL-2 (Figures 2D and 2E). Taken together, these results suggest that within the limits of acceptable toxicity, higher doses of IL-2 might be preferable to lower doses IL-2 in clinical trials of CpG-containing vaccines combined with systemic IL-2.

Regardless of IL-2 dose, most of the OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ T cells elicited by OVA₂₅₇₋₂₆₄+CpG+IFA vaccines were CD62L-negative, CD127-negative effector T cells. Effector CD8⁺ T cells exhibit high levels of direct cytotoxicity, which might be an important attribute for anti-cancer immunity [27].

We found that twenty-eight days after the final vaccination, OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes were detectable in the peripheral blood; however, there was no significant difference in the number of OVA₂₅₇₋₂₆₄-K^b tetramer-binding CD8⁺ lymphocytes in the peripheral blood of mice that were either treated with low dose sc IL-2 or not treated with IL-2. This result is in contrast to our previous work that demonstrated a clear augmentation of splenic TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses by IL-2 when responses were measured twenty-one days after the final vaccination [4].

Importantly for further clinical development, TRP-2₁₈₀₋₁₈₈+CpG-containing vaccines combined with low-dose IL-2 mediated epitope-specific tumor growth inhibition of the poorly immunogenic B16F1 melanoma. In this model, no difference in anti-tumor efficacy was detected when TRP-2₁₈₀₋₁₈₈+CpG-containing vaccines were combined with either high-dose ip IL-2 or low-dose sc IL-2 (Figure 5A).

Our results demonstrate several factors that can affect the magnitude of CD8⁺ T cell responses generated by peptide+CpG-containing vaccines. While CpG-containing peptide vaccines have already been shown to generate large CD8⁺ T cell responses in clinical trials [14], augmentation of these vaccines with IL-2, optimization of vaccination schedules, and increasing the peptide dose in the vaccines might lead to even larger vaccine-elicited CD8⁺ T cell responses and perhaps to greater clinical efficacy of these vaccines.

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Figure 1.

(A) TRP-2₁₈₀₋₁₈₈ in IFA administered subcutaneously for 4 weekly doses elicited a mean TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell response of 0.06% of CD8⁺ T cells when measured by ICCS assay 5 days after the last vaccination. The mean TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell response detected in naïve mice was 0.01% of CD8⁺ T cells (TRP-2₁₈₀₋₁₈₈ in IFA n=7, naïve n=14). (B) The complete vaccination regimen consisted of a series of three priming vaccines administered on days 0, 3, and 6 followed by a boost vaccine on day 21. GM-CSF was administered sc at the vaccine site before each vaccine injection. Complete priming vaccines included 50 μ g TRP-2₁₈₀₋₁₈₈, 60 μ g HBC₁₂₈₋₁₄₀, and 50 μ g CpG in IFA. Complete boost vaccines consisted of the same ingredients except the TRP-2₁₈₀₋₁₈₈ dose was 100 μ g and the

HBC₁₂₈₋₁₄₀ dose was 120 µg. Mice were sacrificed on day 26. Splenocytes were stimulated for six hours with either TRP-2₁₈₀₋₁₈₈ or OVA₂₅₇₋₂₆₄, and ICCS for IFN γ was performed. The plots are gated on CD3⁺ cells. The numbers on the plots show the fraction of CD3⁺CD8⁺ T cells that were IFN γ ⁺. (C) In mice that received TRP-2₁₈₀₋₁₈₈-containing complete vaccination regimens as described in 1B, 3.4% of CD3⁺CD8⁺ splenocytes produced IFN γ in response to TRP-2₁₈₀₋₁₈₈, but only 0.1% of CD3⁺CD8⁺ splenocytes produced IFN γ in response to OVA₂₅₇₋₂₆₄; therefore, the mean TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell response was 3.3%. (n=10 mice per group). (D) One group of mice was vaccinated using the complete vaccination regimen as described in Figure 1B. These mice received priming vaccinations on days 0, 3, and 6 followed by a boost vaccination on day 21. A second group received four vaccinations that were administered on a weekly schedule. The first three vaccinations that were administered to mice receiving weekly vaccines were identical to the complete priming vaccinations and the fourth vaccination was identical to the complete boost vaccinations. All vaccinations in both groups were preceded by GM-CSF injections at the vaccine site. Mice that were vaccinated on both schedules received their final vaccination on day 21. TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses were measured by ICCS on day 26 (Prime-boost n=10, Weekly n=12). (E) Two groups of mice received priming vaccinations on days 0, 3, and 6. Boost vaccinations were administered to both groups on day 21. Both priming and boost vaccinations contained TRP-2₁₈₀₋₁₈₈, HBC₁₂₈₋₁₄₀, and CpG in IFA. The full dose group received priming vaccinations that contained 50 µg of TRP-2₁₈₀₋₁₈₈ and boost vaccines that contained 100 µg of TRP-2₁₈₀₋₁₈₈. The low dose group received vaccinations that contained one-tenth of the full dose of TRP-2₁₈₀₋₁₈₈ (5 µg of TRP-2₁₈₀₋₁₈₈ in priming vaccinations and 10 µg of TRP-2₁₈₀₋₁₈₈ in boost vaccinations). None of the mice in these experiments received GM-CSF. TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses were measured by ICCS assay on day 26 (full dose n=13, low dose n=10). (F) Mice received the complete vaccination regimen described in Figure 1B, which included TRP-2₁₈₀₋₁₈₈+CpG+HBC₁₂₈₋₁₄₀+IFA and GM-CSF or regimens in which one adjuvant at a time was omitted. Only omission of CpG caused a statistically significant decrease in the TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell response (n=10-11 mice per group). TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses were measured by ICCS as described in Figure 1B.

Figure 2.

(A) Mice were vaccinated on days 0, 3, and 6 with vaccines containing 50 μ g TRP-2 $_{180-188}$ and 10 μ g of CpG in IFA. 30,000 IU of IL-2 was administered sc daily on days 7–10 to one group while a second group received control injections. TRP-2 $_{180-188}$ -specific CD8 $^{+}$ T cell responses were measured by ICCS assay as described in Figure 1B on day 13. The plots are gated on CD3 $^{+}$ cells. The numbers on the plots show the fraction of CD3 $^{+}$ CD8 $^{+}$ T cells that were IFN γ^{+} . Low-dose IL-2 dramatically enhanced the percentage (B) and the absolute number (C) of TRP-2 $_{180-188}$ -specific CD8 $^{+}$ T cells elicited by CpG-containing vaccines (IL-2 $n=10$, No IL-2 $n=8$). (D) Two groups of mice were vaccinated as described in 2A. A low-dose group was treated with 30,000 IU of IL-2 sc one time per day on days 7–9. A high-dose group received

40,000 IU of IL-2 ip two times per day on days 7–9. When measured by ICCS assay on day 13, mice that received high-dose ip IL-2 had larger TRP-2_{180–188}-specific CD8⁺ T cell responses than mice that received low-dose sc IL-2. Responses were measured as a percentage of total CD8⁺ T cells (D) and as an absolute number (E) (n=8 mice per group).

Figure 3.

IL-2 increases peripheral blood CD8⁺ T cell responses elicited by CpG-containing vaccines. (A) Two groups of mice were vaccinated on days 0, 3, and 6 with OVA₂₅₇₋₂₆₄+CpG+IFA. One of the groups received 30,000 IU of IL-2 once daily sc on days 7–10. The second group did not receive IL-2. On day 13, blood was collected and tetramer staining was performed. Examples of OVA₂₅₇₋₂₆₄-K^b tetramer staining of peripheral blood lymphocytes (PBL) from one mouse that received IL-2 and one mouse that did not receive IL-2 are shown. The plots are gated on lymphocytes. An example of OVA₂₅₇₋₂₆₄-K^b tetramer staining of PBL from a control mouse is also shown to demonstrate background tetramer staining in this experiment. The numbers on the plots are the percentages of CD8⁺ lymphocytes that bound tetramer. The

control vaccinated mouse was vaccinated with the irrelevant peptide NP₃₆₆₋₃₇₄ plus CpG in IFA and then treated with IL-2. (B) Mice were vaccinated with OVA₂₅₇₋₂₆₄+CpG+IFA as in 3A. One group then received 40,000 IU of IL-2 ip two times per day on days 7–10 while a second group did not receive IL-2. On day 13, blood was collected and tetramer staining was performed. Examples of OVA₂₅₇₋₂₆₄-K^b tetramer staining of PBL from one mouse that received IL-2 and one mouse that did not receive IL-2 are shown. The plots are gated on lymphocytes. An example of OVA₂₅₇₋₂₆₄-K^b tetramer staining of PBL from a control mouse is shown to demonstrate the background tetramer staining in this experiment. The control mouse was vaccinated with the irrelevant peptide E7₄₉₋₅₇ plus CpG in IFA and treated with IL-2. (C) Mice were vaccinated with OVA₂₅₇₋₂₆₄+CpG+IFA on days 0, 3, and 6 as in 3A. One group received 30,000 IU of IL-2 sc once daily on days 7–10 while a second group did not receive IL-2. Mice that received IL-2 had more OVA₂₅₇₋₂₆₄-K^b tetramer⁺ CD8⁺ lymphocytes per μ l of blood than mice that did not receive IL-2 when responses were measured on day 13 (IL-2 n=14, No IL-2 n=13; note log scale on y-axis). (D) Mice were vaccinated with OVA₂₅₇₋₂₆₄+CpG+IFA on days 0, 3, and 6 as in 3A. One group received 40,000 IU of IL-2 two times per day on days 7–10 while a second group did not receive IL-2. Mice that received IL-2 had more OVA₂₅₇₋₂₆₄-K^b tetramer⁺ CD8⁺ lymphocytes per μ l of blood than mice that did not receive IL-2 when responses were measured on day 13 (IL-2 n=9 mice, No IL-2 n=8 mice; note log scale on y-axis). (E) Representative examples are shown of CD127 and CD62L staining of PBL from vaccinated mice that were treated with either low dose or high dose IL-2. The plots are gated on lymphocytes and on CD8⁺ OVA₂₅₇₋₂₆₄-K^b tetramer⁺ events. The numbers on the plots are the percentages of the gated cells in the indicated quadrants. The low-dose IL-2 example is the same mouse labeled low-dose IL-2 in 3A. The high dose IL-2 example is the same mouse labeled high-dose IL-2 in 3B.

Figure 4.

Low-dose sc IL-2 increases peripheral blood TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cell responses. (A) Mice were vaccinated on days 0, 3, and 6 with TRP-2₁₈₀₋₁₈₈+CpG in IFA. The mice were divided into two groups and either treated with IL-2 or not treated with IL-2. On day 13, blood was collected and CD8⁺ T cell responses were measured by ICCS assay. A representative example of the results of an ICCS assay performed on cells from a vaccinated mouse that received 30,000 IU of IL-2 sc daily on days 7–10 and a representative example of the results of an ICCS assay performed on cells from a vaccinated mouse that did not receive IL-2 are shown. The plots are gated on CD3⁺ lymphocytes. The numbers on the plots are the percentages of CD3⁺CD8⁺ lymphocytes that produced IFN γ in response to stimulation with the indicated

peptide. (B) Low-dose sc IL-2 increased the percentage of CD8⁺ peripheral blood T cells that were TRP-2₁₈₀₋₁₈₈-specific (n=8 mice per group). The mice were vaccinated and IL-2 was administered as described in 4A. (C) In the same mice described in 4B, low-dose sc IL-2 increased the absolute number of TRP-2₁₈₀₋₁₈₈-specific CD8⁺ T cells per μ l of blood (n=8 mice per group).

Figure 5.

TRP-2₁₈₀₋₁₈₈+CpG in IFA vaccines combined with low dose IL-2 inhibit B16F1 tumor growth. Mice were injected with 30,000 B16F1 tumor cells on day 0 and vaccinated with peptide+CpG in IFA vaccines on days 0, 3, and 6. The vaccines included either the tumor-associated peptide TRP-2₁₈₀₋₁₈₈ or the negative control peptide E7₄₉₋₅₇. All mice that received E7₄₉₋₅₇+CpG in IFA vaccines received 30,000 IU of IL-2 sc daily on days 7–9 (low-dose IL-2). Mice that received TRP-2₁₈₀₋₁₈₈+CpG in IFA vaccines received either 30,000 IU of IL-2 sc once daily on days 7–9 (low-dose IL-2) or 40,000 IU of IL-2 two times per day ip on days 7–9 (high-dose IL-2). (A) Regardless of IL-2 dose, mice that received TRP-2₁₈₀₋₁₈₈-containing vaccines had smaller tumors (P<0.004) than mice that received E7₄₉₋₅₇-containing

vaccines at the indicated (*) time points. There was no difference in tumor size between TRP-2₁₈₀₋₁₈₈-vaccinated mice that received either high-dose or low-dose IL-2 (TRP-2₁₈₀₋₁₈₈-vaccinated+low-dose IL-2 n=12, TRP-2₁₈₀₋₁₈₈-vaccinated+high-dose IL-2 n=12, E7₄₉₋₅₇-vaccinated+low-dose IL-2 n=13). (B) Mice were injected with 30,000 B16F1 tumor cells on day 0. Mice that received TRP-2₁₈₀₋₁₈₈+CpG in IFA vaccines on days 0, 3, and 6 plus 30,000 IU of IL-2 sc once daily on days 7–9 had increased survival compared to mice that were treated identically except that their vaccines included the negative control peptide E7₄₉₋₅₇ in place of TRP-2₁₈₀₋₁₈₈ (TRP-2₁₈₀₋₁₈₈-vaccinated n=12, E7₄₉₋₅₇-vaccinated n=13).