MINIREVIEW

A Comparison and Critical Analysis of Preclinical Anticancer Vaccination Strategies

J. N. KOCHENDERFER[1] AND R. E. GRESS

Experimental Transplantation and Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Anticancer vaccines have been extensively studied in animal models and in clinical trials. While vaccination can lead to tumor protection in numerous murine models, objective tumor regressions after anticancer vaccination in clinical trials have been rare. B16 is a poorly immunogenic murine melanoma that has been extensively used in anticancer vaccination experiments. Because B16 has been widely used, different vaccination strategies can be compared. We reviewed the results obtained when B16 was treated with five common vaccine types: recombinant viral vaccines, DNA vaccines, dendritic cell vaccines, whole-tumor vaccines, and peptide vaccines. We also reviewed the results obtained when B16 was treated with vaccines combined with adoptive transfer of tumor antigen–specific T cells. We found several characteristics of vaccination regimens that were associated with antitumor efficacy. Many vaccines that incorporated xenogeneic antigens exhibited more potent anticancer activity than vaccines that were identical except that they incorporated the syngeneic version of the same antigen. Interleukin-2 enhanced the antitumor efficacy of several vaccines. Finally, several effective regimens generated large numbers of tumor antigen–specific $CD8^+$ T cells. Identification of vaccine characteristics that are associated with antitumor efficacy may aid in the development of more effective anticancer vaccination strategies. Exp Biol Med 232:1130–1141, 2007

This work was supported by intramural funding from the National Cancer Institute.

[1] To whom correspondence should be addressed the National Institutes of Health, 10 Center Drive, CRC 3-3288, Bethesda, MD 20892. E-mail: kochendj@mail.nih.gov

Received February 25, 2007.
Accepted June 13, 2007.

DOI: 10.3181/0702-MR-42
1535-3702/07/2329-1130$15.00

Key words: vaccination; tumor immunity; peptides; cytokines; T cells; adoptive T-cell therapy; CpG oligodeoxynucleotides

Introduction

Anticancer vaccination strategies that have been tested in clinical trials have only rarely been associated with objective tumor regressions (1). More effective anticancer vaccination regimens are clearly needed. In an attempt to develop more effective anticancer vaccines, many investigators have tested vaccination strategies in murine models. Because a wide variety of murine tumor models have been used to test vaccination strategies, comparison of the efficacy of different strategies is difficult. In addition, some tumor cell lines that have been used to test anticancer vaccines have been genetically altered to express highly immunogenic foreign proteins, possibly making them more susceptible than most human tumors to immunologic therapies.

B16 is a murine tumor cell line that was derived from a spontaneously arising melanoma of C57BL/6 origin (2). B16 is poorly immunogenic, and it expresses very low levels of major histocompatibility complex (MHC) class I molecules, making it difficult for $CD8^+$ T cells to recognize (3, 4). Because B16 has been widely studied, several different vaccination strategies using it can be compared. In addition, several antigens expressed by B16 have been molecularly defined, allowing careful measurement of antigen-specific immune responses. Tyrosinase-related protein-2 (TRP-2; Ref. 2) and gp100 (5) are proteins expressed by normal murine melanocytes and B16 that contain immunogenic MHC class I–presented epitopes. TRP-1 is a

Table 1. Advantages and Disadvantages of Different Vaccination Strategies

Strategy	Advantages	Disadvantages	References
Viral vaccines	$CD8^+$ T-cell and antibody responses can be generated against whole proteins.	Neutralizing immune responses against viral proteins limit the ability to perform repeated immunizations.	6, 8, 12, 13, 15, 16
DNA vaccines	Easy to manufacture. In mice, $CD8^+$ T-cell and antibody responses can be generated against whole proteins.	May be less effective than viral or peptide vaccines at inducing $CD8^+$ T-cell responses.	17–22
Dendritic cell vaccines	Cause impressive antitumor immunity in murine models.	Generation of dendritic cells is technically challenging. Anti–fetal bovine serum immune responses might make dendritic cells more effective in mouse models than in clinical trials.	23–29
Whole-tumor cell vaccines	Use of autologous tumor cells as vaccines potentially allows generation of immune responses against all of the proteins expressed by the tumor, including proteins with tumor-specific mutations.	Obtaining large numbers of tumor cells so that clinical trials accurately mimic mouse models is difficult.	10, 30–33
Peptide vaccines	Easy to manufacture. Strong $CD8^+$ T-cell responses can be generated with repetitive vaccination or by using adjuvants like CpG oligodeoxynucleotides.	Immune responses limited to one or a few epitopes.	9, 34–40, 45
Vaccines combined with adoptive T-cell therapy	The most effective strategy for treating established B16.	The most effective murine model of adoptive T-cell transfer combined with vaccination used transgenic T cells; therefore, it cannot be perfectly copied in clinical trials.	14, 24, 54–56, 61

cell surface protein that can be the target of antibodies against B16 (6).

In this review, we critically analyze and compare different anticancer vaccination strategies that have been used to treat B16 in syngeneic mice. Analyzing and comparing the different treatment strategies might help identify important features of effective therapies and point toward the most promising approaches for future investigation. We will first review the results obtained when B16 was treated with several different anticancer vaccination strategies: recombinant viral vaccines, DNA vaccines, dendritic cell (DC) vaccines, whole-tumor vaccines, peptide vaccines, and vaccines combined with adoptive T-cell transfer (Table 1). Next, we will try to define the characteristics of anticancer vaccination strategies that were critical for generation of anticancer immunity.

Vaccination with Recombinant Viruses

Natural viral infections elicit potent immune responses that can clear acute infections and generate long-term immunologic memory (7). Advances in molecular biology have made it possible to construct recombinant viruses that encode the genes for tumor antigens. Several recombinant viral vaccines have been used to treat B16.

Extensive work has been performed using recombinant adenoviruses encoding the gene for the TRP-2 protein. When mice were vaccinated with a recombinant adenovirus encoding the gene for human TRP-2 and then challenged intravenously (iv) with B16, all mice were completely protected from pulmonary tumor formation, and 50% of mice that were vaccinated with a recombinant adenovirus encoding human TRP-2 were protected from a subcutaneous (sc) tumor challenge (8). In contrast, when mice were vaccinated with an adenovirus encoding murine TRP-2 and then challenged with B16, the reduction in lung tumor formation was much less than that observed when mice were vaccinated with adenovirus encoding human TRP-2 (8). In addition, vaccination with adenovirus encoding murine TRP-2 did not prevent sc tumor formation in any mice (8). Amino acids 180–188 of TRP-2 ($TRP\text{-}2_{180–188}$) form an immunogenic MHC class I–restricted epitope (9, 10). Interestingly, amino acids 180–188 of TRP-2 are identical in mice and humans, and these amino acids form a human leukocyte antigen-A2 (HLA-A2)–restricted epitope that can elicit $CD8^+$ T-cell responses in humans (11). Despite the identical sequence of this immunogenic epitope in the murine and human versions of TRP-2, vaccination with adenovirus encoding the gene for human TRP-2 elicited $CD8^+$ T-cell responses against $TRP\text{-}2_{180–188}$, but vaccination with adenovirus encoding the murine version of

TRP-2 did not (8). Vaccination with recombinant adenovirus encoding human TRP-2 led to loss of fur pigmentation consistent with vitiligo, but vaccination with adenovirus encoding murine TRP-2 did not cause a loss of fur pigmentation (8). Notably, vaccination with recombinant adenoviruses encoding human TRP-2 was more effective than vaccination with plasmid DNA encoding the same gene at protecting mice from a B16 challenge (8). A CD8-dependent therapeutic effect was observed when a recombinant adenovirus encoding human TRP-2 was used to treat B16 that had been injected sc 3 days previously (12). In contrast, vaccination with recombinant adenovirus encoding murine TRP-2 was not effective at treating B16 that had been injected 3 days earlier (12). In mice vaccinated with recombinant adenovirus encoding human TRP-2, $CD8^+$ T cells specific for $TRP\text{-}2_{180–188}$ made up 1% of total $CD8^+$ T cells (12).

As detailed above, vaccination with recombinant adenovirus encoding human TRP-2 was much more effective than vaccination with adenovirus encoding the murine version of TRP-2 at mediating protection against a B16 challenge. Steitz *et al*. hypothesized that the enhanced tumor protection observed with vaccination using adenovirus encoding human TRP-2 compared with adenovirus encoding murine TRP-2 was due to the presence of foreign MHC class II–presented epitopes that are capable of eliciting a $CD4^+$ helper T-cell response in the human but not the murine version of TRP-2. To test the hypothesis that foreign helper epitopes can enhance vaccination with TRP-2, mice were vaccinated with recombinant adenovirus encoding murine TRP-2 fused to jellyfish enhanced green fluorescent protein (EGFP-murine TRP-2; Ref. 13). Vaccination with adenovirus encoding EGFP-murine TRP-2 was as effective as vaccination with adenovirus encoding human TRP-2 in preventing lung tumor formation in mice challenged iv with B16 (13). As in previous experiments, vaccination with adenovirus encoding murine TRP-2 without EGFP was not effective at protecting mice from a B16 challenge (13). In the work described above (8, 12, 13), all of the vaccines that were effective therapy for B16 incorporated the human form of the TRP-2 protein. Using xenogeneic forms of self-antigens for vaccination may lead to more potent immune responses than vaccination with the self-antigen.

Very effective protection against a sc B16 challenge as well as extensive depigmentation of fur consistent with vitiligo was induced by vaccination with recombinant vaccinia virus encoding the gene for the melanocyte cell surface protein murine TRP-1 (6). Vaccination with recombinant vaccinia virus encoding murine TRP-1 elicited high-titer IgG2b antibodies specific for TRP-1. Experiments using MHC class II–deficient mice, which lack $CD4^+$ T cells, showed that antibody production and protection from tumor challenge were clearly dependent on $CD4^+$ T cells (6). In addition, depigmentation consistent with vitiligo was not observed when MHC class II–deficient mice were vaccinated. In contrast, β_2-microglobulin–deficient mice, which lack $CD8^+$ T cells, were protected from B16 challenge to the same degree as wild-type mice (6). Antibodies might be especially important in the treatment of tumors that express low levels of MHC class I molecules, such as B16 (3, 4).

Vaccination with recombinant vaccinia or recombinant fowlpox viruses encoding the gene for either the human or murine version of the melanocyte-associated protein gp100 were not effective therapies for B16 (14). However, vaccination with recombinant vaccinia or recombinant fowlpox viruses encoding the gene for human gp100 is an essential component of a very effective treatment regimen for B16 that also includes adoptive transfer of gp100-specific $CD8^+$ T cells and administration of interleukin-2 (IL-2; Ref. 14). This regimen will be described in detail later in this review.

Viral vaccines have elicited $CD8^+$ T-cell responses against tumor-associated antigens in human clinical trials, but objective antitumor responses attributable to viral vaccines have been rare (1, 15, 16). One factor that might limit the effectiveness of viral vaccines is the generation of neutralizing immune responses against viral proteins (15).

DNA Vaccines

Immunization with plasmid DNA can elicit antigen-specific immune responses consisting of $CD8^+$ T cells, $CD4^+$ T cells, and antibodies (17). DNA vaccination has been evaluated in B16 therapy and prophylaxis experiments (18–20). In most studies of DNA vaccination that used B16, the DNA was administered by the gene gun method of immunization. In gene gun immunization, plasmid DNA is coated onto gold particles followed by delivery of the DNA-coated particles into the skin by a high-pressure, helium-driven gene gun (20).

Gene gun immunization with plasmid DNA encoding human but not murine TRP-1 protected mice from lung tumor formation after an iv challenge with B16 (20). Additionally, immunization with DNA encoding human but not murine TRP-1 caused a loss of fur pigmentation consistent with vitiligo (20). Tumor protection was dependent on $CD4^+$ T cells, NK cells, and the Fc-receptor γ–chain (20). Immunization with human TRP-1, but not murine TRP-1, elicited antibodies against murine TRP-1 (20).

DNA vaccination by gene gun delivery of plasmid DNA encoding human but not murine TRP-2 protected mice against lung tumor formation after an iv challenge with B16 (18). The antitumor effect was dependent on both $CD8^+$ and $CD4^+$ T cells, because protection was abrogated in both β2-microglobulin–deficient mice, which lack $CD8^+$ T cells, and in MHC class II–deficient mice, which lack $CD4^+$ T cells (18). Depigmentation consistent with vitiligo occurred after vaccination with human but not murine TRP-2 (18). This depigmentation was also dependent on both $CD4^+$ and $CD8^+$ T cells. Vaccination with human TRP-2

DNA elicited a $CD8^+$ T-cell response against an epitope of murine TRP-2, but vaccination with murine TRP-2 DNA did not elicit a T-cell response against this epitope (18).

Administration of plasmid DNA encoding the gene for IL-12 along with a second plasmid encoding murine TRP-2 protected 40% of mice against an sc B16 challenge (19). The mechanism of protection against B16 challenge mediated by administration of DNA encoding murine TRP-2 and IL-12 was not determined (19). Gene gun immunization with DNA encoding murine TRP-2 without the IL-12 gene was not effective at protecting against a B16 challenge (19). As with viral vaccinations (8, 12, 13), most of the studies that achieved an antitumor effect against B16 with DNA vaccines used plasmid DNA that encoded the xenogeneic form of self-antigens (18, 20). Two reports have suggested that DNA vaccination is a less effective therapy for B16 than other types of vaccination (8, 21). Human clinical trials have assessed the ability of DNA vaccination, viral vaccination, and peptide-in-adjuvant vaccination to induce $CD8^+$ T-cell responses against a tumor-associated antigen (22). Vaccination with DNA did not induce detectable $CD8^+$ T-cell responses, but detectable $CD8^+$ T-cell responses were elicited by viral vaccination and peptide-in-adjuvant vaccination (22).

DC Vaccines

Several groups have treated B16 by vaccinating mice with DCs. The DCs were loaded with either MHC class I–presented peptides (21, 23, 24) or tumor lysate (25), or were transduced with adenovirus encoding a tumor-associated antigen (26). The DCs used in all of these studies were derived by culturing bone marrow cells *in vitro* with cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α; Refs. 21, 23–25). A series of three vaccinations with DCs that were loaded with a peptide from TRP-2 protected 35% of mice that were challenged sc with B16 following completion of the vaccinations (23). The same regimen caused rejection of B16 tumor cells injected sc on the day prior to the initiation of vaccination in 20% of mice (23). When Schreurs *et al*. vaccinated mice with TRP-$2_{180–188}$ peptide–pulsed DCs and then challenged the mice sc with B16, 80% of the mice rejected the tumor challenge (21). These same authors also vaccinated mice with the TRP-$2_{180–188}$ peptide emulsified in incomplete Freund adjuvant (IFA). The peptide emulsified in IFA was completely ineffective at mediating tumor protection (21). In addition, these authors vaccinated mice with plasmid DNA encoding the TRP-2 protein. DNA vaccination was also completely ineffective at mediating tumor protection (21). DCs pulsed with tumor lysates made from B16 cells have been shown to protect mice against a challenge with live B16 cells (25). Tumor protection mediated by tumor lysate–loaded DCs was enhanced by loading the DCs with keyhole limpet hemocyanin (KLH) in addition to tumor lysate (25). KLH is a protein that is known to stimulate helper T-cell responses. Administration of systemic IL-2 to mice that received tumor lysate–pulsed DCs also enhanced tumor protection (25). Vaccination with DCs loaded with an immunogenic peptide from the gp100 protein has been shown to enhance therapy of established B16 tumors by adoptively transferred T cells that were specific for the same gp100 peptide with which the DCs cells were loaded (24).

One critical factor that must be kept in mind when evaluating all of the DC experiments described above is that the DCs used in these experiments were derived by *in vitro* culture in media containing fetal bovine serum (FBS; Refs. 21, 23–25). The B16 cells used in the *in vivo* tumor challenge experiments were also cultured in media that contained FBS (21, 23–25). Because the DCs and the tumor cells were both cultured in media that contained FBS, immune responses elicited by the DC vaccines against components of the FBS might have caused an antitumor effect. DCs derived from bone marrow cells by culture in media that contained FBS could mediate protection against an sc or an iv challenge with the B78-D14 variant of B16, regardless of whether the DCs were loaded with a tumor-associated antigen (27). The level of protection achieved by the unloaded FBS–cultured DCs was almost equal to that observed with similarly cultured DCs that were loaded with the TRP-$2_{180–188}$ peptide (27). Epidermal Langerhans cells, which share many characteristics with bone marrow–derived DCs, can be obtained without culture in media containing FBS. In contrast to DCs that were cultured in media containing FBS, vaccination with epidermal Langerhans cells that were not exposed to FBS led to protection against B78-D14 only when the epidermal Langerhans cells were loaded with TRP-$2_{180–188}$ (27). Systemic IL-2 therapy was required for the epidermal Langerhans cells loaded with TRP-$2_{180–188}$ to protect mice from a tumor challenge (27). Similar experiments conducted by other investigators have also demonstrated an antitumor effect of FBS-cultured DCs that were not loaded with any tumor antigens against B16 cells that were cultured in FBS (28). DCs cultured in FBS might stimulate an anti-FBS immune response that under some conditions is able to eliminate tumor cells that have been cultured in FBS (27, 28).

Ribas and coworkers vaccinated mice with DCs that were transduced with replication-deficient adenoviral vectors encoding the cDNA for the human melanoma antigen recognized by T cells-1 (MART-1; Ref. 26). After receiving a single injection of MART-1–transduced DCs, 23.5% of mice were protected from a subsequent sc challenge with B16 (26). In contrast, vaccination with control-transduced DCs caused minimal inhibition of B16 growth (26). The B16 cells used in these experiments were obtained from tumors growing in syngeneic mice (26). This experimental design eliminated the confounding factor of culturing both DCs and B16 in FBS-containing media. In contrast to vaccination with MART-1–transduced DCs, direct vacci-

nation of mice with adenovirus encoding the cDNA for MART-1 did not protect against a subsequent challenge with B16 (26). The protection against B16 mediated by MART-1–transduced DCs was dependent on natural killer cells (29).

Whole-Tumor Vaccines

Because B16 is a poorly immunogenic tumor, vaccination with irradiated wild-type B16 tumor cells does not protect mice from a subsequent challenge with live B16 cells (30). Dranoff and coworkers transduced B16 with the gene for GM-CSF. The cells then continuously secreted GM-CSF. These B16–GM-CSF cells were used as a vaccine by irradiating them and injecting them into mice sc (30). Vaccination with B16–GM-CSF cells could protect mice from a subsequent challenge with live, wild-type B16 (30).

One of the most successful approaches for treatment of B16 has been vaccination with irradiated B16–GM-CSF cells combined with blockade of cytotoxic T-lymphocyte antigen-4 (CTLA-4; Refs. 10, 31, 32). When CTLA-4 on T cells binds to the costimulatory molecules B7–1 and B7–2 on antigen-presenting cells (APCs), T-cell responses are inhibited. Certain antibodies can block the interaction of CTLA-4 and the B7 molecules, leading to an enhancement of T-cell responses (32). In tumor therapy experiments, a series of three injections of irradiated B16–GM-CSF cells followed by treatment with a CTLA-4 blocking antibody resulted in long-term survival of 80% of mice that were injected with live wild-type B16 cells on the same day as initiation of B16–GM-CSF vaccination (32). This impressive antitumor immunity was dependent on $CD8^+$ T cells and NK cells (32). In this therapeutic model, mice treated with only B16–GM-CSF vaccination exhibited a modest degree of tumor growth inhibition, and mice treated with only anti–CTLA-4 were not protected from a B16 challenge at all (32). TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses that made up greater than 1% of total $CD8^+$ T cells were detected *ex vivo* by tetramer and intracellular cytokine staining analyses in mice that were vaccinated with B16–GM-CSF and treated with anti–CTLA-4 (10, 31). Some of the mice treated with B16–GM-CSF plus anti–CTLA-4 developed fur depigmentation consistent with autoimmune destruction of melanocytes (10, 32). Moreover, histologic evidence of autoimmune destruction of melanocytes was detected at sites of fur depigmentation (10). In tumor prophylaxis experiments, tumor protection caused by B16–GM-CSF plus anti–CTLA-4 involved redundant mechanisms, because 100% of mice were protected from a challenge with wild-type B16, despite depletion of natural killer cells or $CD8^+$ T cells or $CD4^+$ T cells (10). This is in contrast to earlier work in which mice were vaccinated with B16–GM-CSF without anti–CTLA-4 and then later challenged with wild-type B16 (33). In these experiments, rejection of a B16 challenge by vaccination with B16–GM-CSF alone was completely dependent on $CD4^+$ T cells, and rejection of a B16 challenge was partially impaired in the absence of $CD8^+$ T cells (33). Treatment of mice with the anti-CD25 antibody PC61, which binds to $CD4^+CD25^+$ regulatory T cells, was shown to increase the antitumor efficacy of a regimen of B16–GM-CSF plus anti–CTLA-4 (31).

In mice, vaccination with B16–GM-CSF combined with anti–CTLA-4 is a very effective therapy for B16. Because the whole-tumor cell is used as a vaccine, any antigen expressed by the tumor, including unique tumor-specific mutated proteins, might elicit an immune response. These immune responses might involve T cells or B cells. Accurately mimicking the murine whole-tumor vaccination studies in humans requires either a large number of primary tumor cells or an immortalized tumor cell line from each patient to be vaccinated.

Peptide Vaccines

Vaccines consisting of peptides emulsified in IFA are easy to manufacture and have been nontoxic in multiple clinical trials (34–36). Repetitive vaccination with peptides in IFA can generate large numbers of epitope-specific $CD8^+$ T cells (37), but peptides in IFA vaccines have only rarely caused objective antitumor responses (1). Vaccination of mice with the TRP-$2_{180–188}$ peptide in IFA was not effective at causing inhibition of B16 growth (21), and vaccination with TRP-$2_{180–188}$ in IFA elicited very small $CD8^+$ T-cell responses (38, 39). The size of epitope-specific $CD8^+$ T-cell responses elicited by peptide in IFA vaccines was dramatically increased by the addition of oligodeoxynucleotides containing unmethylated CpG motifs (CpGs; Refs 9, 38–40). CpGs have been divided into three classes: class A CpGs, class B CpGs, and class C CpGs (41, 42). Compared with class A CpGs, class B CpGs generally cause more B-cell proliferation but less interferon-alpha secretion (41, 42). Class C CpGs induce both B-cell proliferation and interferon-alpha secretion (42). CpG 1826 (43), which is a class B CpG, was used in all of the experiments described in this review. Class B CpGs induce maturation of DCs, which leads to increased costimulatory molecule expression on the DC surface (41). Class B CpGs also increase production of IL-6, IL-12, and TNF-α (41). The mechanism of action of all CpGs involves binding to Toll-like receptor 9 (TLR9; Ref. 42). The cellular distribution of TLR9 differs between humans and mice. Mice express TLR9 on B cells, monocytes, and probably all DCs (42). In contrast, humans only express TLR9 on B cells and the plasmacytoid subset of DCs (41, 42). Despite the difference in TLR9 expression, class B CpGs can enhance T-cell immunization in both humans (42, 44) and mice (9, 38–40).

In order to study epitope-specific antitumor immunity generated by peptide+CpG–containing vaccines, experiments were performed by vaccinating one group of mice with vaccines containing TRP-$2_{180–188}$+CpG and simultaneously vaccinating a second group with vaccines contain-

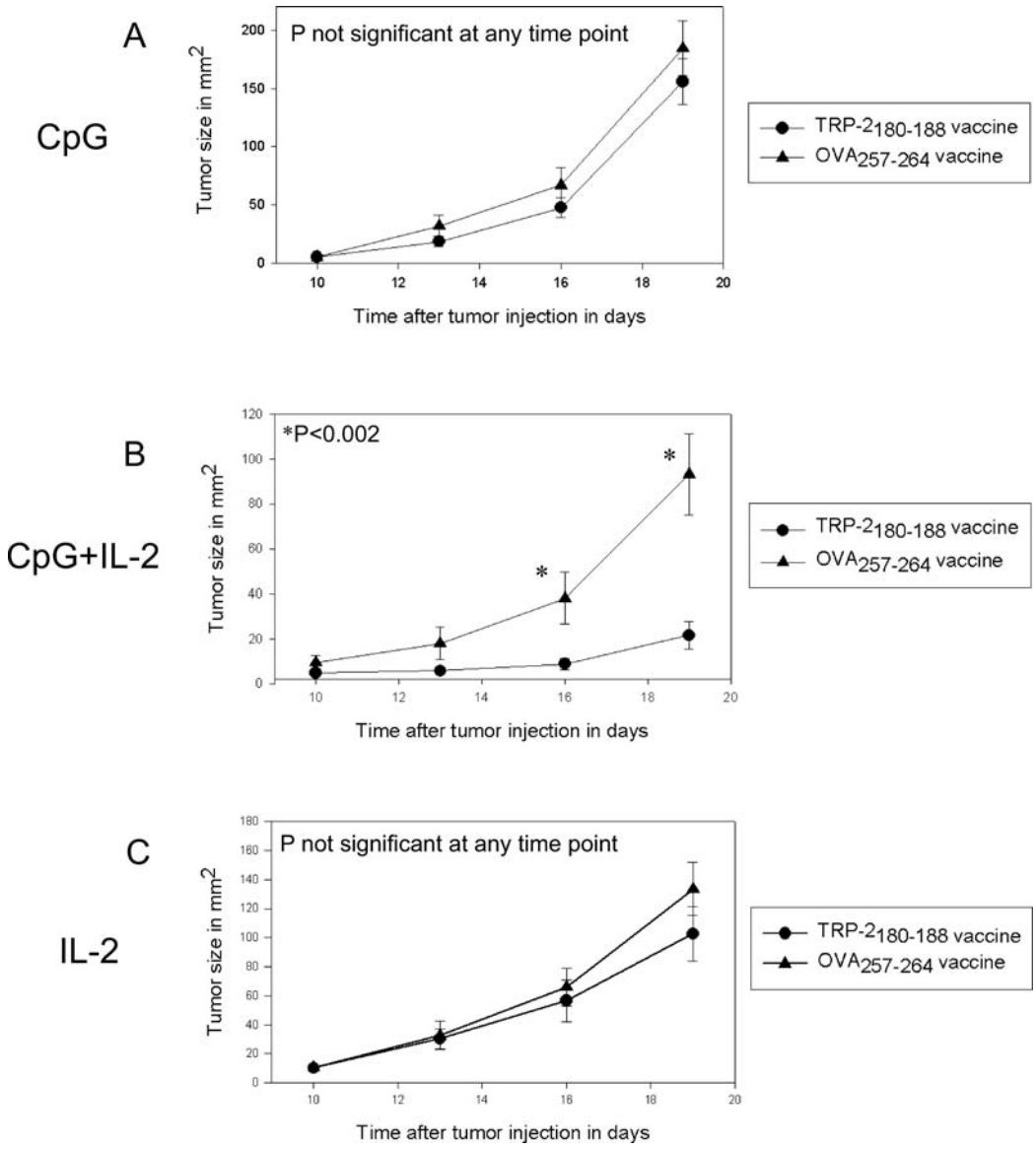

Figure 1. (A) Both IL-2 and CpGs are required for peptide vaccination to inhibit B16 growth. Mice were injected with B16 melanoma on Day 0. Priming vaccinations consisting of peptides+CpG in IFA were administered on Days 0, 3, and 6. A boost vaccine containing peptides+CpG in IFA was administered on Day 14. One group received vaccines containing the tumor-associated peptide TRP-$2_{180–188}$, and another group received vaccines containing the negative control peptide $OVA_{257–264}$. Aside from the different peptides, the two groups were treated identically. Tumors were measured every 3 days starting on Day 10. There was not a statistically significant difference in tumor size when TRP-$2_{180–188}$–vaccinated mice were compared to $OVA_{257–264}$–vaccinated mice (TRP-$2_{180–188}$–vaccinated: $n = 12$, $OVA_{257–264}$–vaccinated: $n = 11$). (B) B16 was injected on Day 0, and mice were vaccinated on Days 0, 3, 6, and 14 in the same manner as in panel A. The mice were administered 40,000 IU of IL-2 intraperitoneally twice daily on Days 7–10 and on Days 15–18. Tumor size was smaller in TRP-$2_{180–188}$–vaccinated mice than in $OVA_{257–264}$–vaccinated mice. A statistically significant difference ($P < 0.002$) between the two groups occurred at the indicated (*) time points. TRP-$2_{180–188}$–vaccinated: $n = 14$, $OVA_{257–264}$–vaccinated: $n = 16$). (C) Mice were injected with B16 on Day 0. Mice received vaccinations and IL-2 as described in panel B, except that CpG was omitted. There was no difference in tumor growth rate when mice receiving TRP-$2_{180–188}$–containing vaccines were compared to mice receiving vaccines containing the $OVA_{257–264}$ negative control peptide. TRP-$2_{180–188}$–vaccinated: $n = 13$, $OVA_{257–264}$–vaccinated: $n = 12$). Note: this figure was originally published in The Journal of Immunology (2006) 177:8861. Copyright 2006 The American Association of Immunologists, Inc. Used with permission.

ing the negative control peptide $OVA_{257–264}$ plus CpG (38). Because both groups were treated identically aside from the difference in peptide, the difference in tumor growth between mice that received vaccines containing TRP-$2_{180–188}$ and those that received vaccines containing $OVA_{257–264}$ was epitope specific and was not due to the activation of non–epitope-specific effector cells, such as natural killer cells (38). Somewhat surprisingly, vaccination with TRP-$2_{180–188}$+CpG–containing vaccines alone did not cause epitope-specific inhibition of B16 growth, but administration of systemic IL-2 with TRP-$2_{180–188}$+CpG–containing vaccines did lead to epitope-specific, CD8-dependent inhibition of B16 growth after mice were challenged with B16 cells on the same day as initiation of vaccination

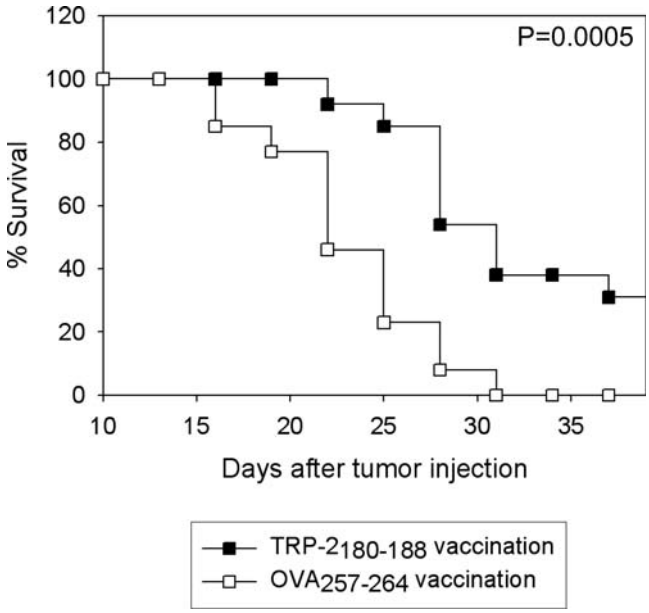

Figure 2. Mice received priming vaccines on Days 0, 3, and 6, and a boost vaccine on Day 14. One group of mice received vaccines containing TRP-$2_{180–188}$+CpG in IFA, and another group received vaccines containing $OVA_{257–264}$+CpG in IFA. IL-2 was administered on Days 7–9 and Days 15–21. All IL-2 doses were 40,000 IU administered intraperitoneally. Aside from the different peptides, the two groups were treated identically. B16 was injected on Day 19. Tumors were measured every 3 days starting on Day 29 (10 days after tumor injection). Mice were euthanized when the product of perpendicular tumor dimensions reached 200 mm^2 or when tumor ulceration developed. Survival was increased in mice that received vaccines containing the tumor-associated peptide TRP-$2_{180–188}$ compared with mice that received vaccines containing the negative control peptide $OVA_{257–264}$ (n = 13 mice per group).

(Fig. 1; Ref. 38). Vaccination with TRP-$2_{180–188}$ in IFA without CpGs combined with administration of IL-2 did not cause epitope-specific tumor growth inhibition (Fig. 1; Ref. 38). The ability to cause epitope-specific tumor growth inhibition correlated with the size of $CD8^+$ T-cell responses elicited by the different vaccination regimens. When $CD8^+$ T-cell responses were measured *ex vivo*, 18% of $CD8^+$ T cells were TRP-$2_{180–188}$ specific after mice were vaccinated with TRP-$2_{180–188}$+CpG–containing vaccines and treated with systemic IL-2 (38). In contrast, only 1.1% of $CD8^+$ T cells were TRP-$2_{180–188}$ specific in mice vaccinated with TRP-$2_{180–188}$+CpG–containing vaccines but not treated with IL-2 (38). Vaccination with TRP-$2_{180–188}$ in IFA without CpGs followed by treatment with IL-2 led to TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses that made up 2.8% of $CD8^+$ T cells (38). TRP-$2_{180–188}$+CpG in IFA vaccination plus systemic IL-2 was also tested in tumor prophylaxis experiments. In a prophylactic model, 23% of mice that were vaccinated with TRP-$2_{180–188}$+CpG-containing vaccines, treated with IL-2, and then challenged with B16 survived tumor free. Mice that were treated in an identical manner, except that the negative control peptide $OVA_{257–264}$ replaced TRP-$2_{180–188}$ in their vaccines, all died soon after tumor challenge (Fig. 2).

Potent anti-B16 immunity was generated by TRP-$2_{180–188}$+CpG in IFA vaccines combined with IL-2 during the period of immune reconstitution after myeloablative doses of total body irradiation and syngeneic bone marrow transplantation (45). Vaccination with TRP-$2_{180–188}$+CpG in IFA combined with administration of systemic IL-2 during early immune reconstitution after bone marrow transplantation elicited TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses that made up 9.1% of $CD8^+$ T cells (45). Generation of these large TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses depended on synergism between CpGs and IL-2 (45). When mice were injected with B16 14 days after bone marrow transplantation, a regimen initiated the same day as B16 injection that consisted of TRP-$2_{180–188}$+CpG in IFA vaccines combined with systemic IL-2 inhibited growth of B16 and increased survival in an epitope-specific manner (45).

A regimen of TRP-$2_{180–188}$ in IFA combined with CpGs and CTLA-4 blockade with an anti–CTLA-4 antibody was able to inhibit growth of B16 in a therapeutic model, but no mice survived long term (9). In addition, this regimen did not protect mice from a B16 challenge in a prophylactic model (9). However, when mice were vaccinated with TRP-$2_{180–188}$ in IFA, treated with CpGs and CTLA-4 blockade prophylactically, then challenged with B16 and treated with a second full course of TRP-$2_{180–188}$ in IFA, CpGs, and CTLA-4 blockade, 80% of mice survived tumor free (9).

The avidity of a T cell is a measure of the ability of a T cell to be activated by graded concentrations of peptide–MHC on APCs (46). A highly avid T cell can recognize APCs expressing a low concentration of peptide–MHC. Conversely, activation of low-avidity T cells requires APCs that express a high concentration of the relevant peptide–MHC. The avidity of vaccine-elicited T cells can be measured by determining the percentage of the maximum peptide-specific T-cell response that can be elicited by stimulating the T cells with APCs that display decreasing concentrations of peptide–MHC (47). Avidity is important, because high-avidity $CD8^+$ T cells have been shown to be more effective than low-avidity $CD8^+$ T cells at eradicating B16 (48). In addition, adoptive transfer of high-avidity $CD8^+$ T cells has been shown to be more effective at inducing autoimmune diabetes than adoptive transfer of low-avidity $CD8^+$ T cells (49). Mechanisms of tolerance eliminate most high-avidity T cells that are specific for antigens expressed by normal host tissues (50–53). Possibly because TRP-2 is a protein that is expressed by normal tissues (2), most TRP-$2_{180–188}$–specific $CD8^+$ T cells that are elicited by peptide vaccination are low-avidity T cells (38). The low avidity of these T cells might limit the antitumor efficacy of vaccines designed to elicit TRP-$2_{180–188}$–specific $CD8^+$ T cells.

Vaccination Combined with Adoptive T-cell Transfer

Most vaccination strategies had only limited success against B16 when vaccination was initiated at the same time

as tumor challenge or following tumor challenge (8, 9, 12, 18, 23, 38). No therapeutic approach consisting of vaccination alone can reliably eradicate palpable B16 tumors. Moreover, anticancer vaccination has rarely led to objective remissions of cancer in clinical trials (1). In an attempt to develop more powerful T-cell therapies against cancer, vaccination has been combined with adoptive transfer of tumor antigen–specific T cells. The first T-cell therapy to consistently eradicate previously established, palpable B16 tumors was a regimen that combined adoptive transfer of gp100-specific $CD8^+$ T cells, vaccination, and administration of systemic IL-2 (14). In order to eradicate B16, all three components of this regimen were required (14). The adoptively transferred cells used in these regimens were obtained from transgenic mice called pmel-1 mice, in which almost all $CD8^+$ T cells express a T-cell receptor (TCR) that is specific for the gp100 tumor antigen (14). The TCR genes used to create pmel-1 mice were obtained from a T-cell clone that was generated by vaccinating a mouse with human gp100 and then further stimulating T cells from this mouse *in vitro* with human gp100 (5). Despite being elicited with human gp100, this T-cell clone recognized the epitope formed by amino acids 28–33 of the murine gp100 protein ($gp100_{28–33}$; Ref. 5). This cross-reactivity occurs despite the fact that murine and human $gp100_{28–33}$ differ at three of nine amino acids (14).

In pmel-1 transgenic mice, almost all $CD8^+$ T cells recognize the gp100 antigen that is expressed by B16 (14). Interestingly, B16 growth is not different in untreated wild-type mice compared with untreated pmel-1 mice (14). B16 tumor regression does take place in pmel-1 mice when they are vaccinated against gp100 and treated with IL-2 (14). These findings show that even very high numbers of tumor antigen–specific $CD8^+$ T cells cannot eradicate poorly immunogenic B16 tumor cells unless the T cells are activated by vaccination and IL-2.

Four different vaccine types have been used successfully in combination with adoptive transfer of pmel-1 cells and IL-2. These regimens mediated antitumor immunity when vaccines included human $gp100_{28–33}$ but not when vaccines included murine $gp100_{28–33}$ (14). In these regimens, the most commonly used vaccines have been recombinant vaccinia viruses or recombinant fowlpox viruses encoding human gp100 (14, 54, 55). A vaccination regimen consisting of the human $gp100_{28–33}$ peptide in IFA followed by injections of an anti-CD40 antibody was also used in conjunction with pmel-1 cells and IL-2 to treat B16 (14). Finally, B16 tumors have been eradicated by transfer of pmel-1 cells combined with administration of IL-2 and vaccination with DCs that were pulsed with the human $gp100_{28–33}$ peptide (24). After myeloablative doses of total body irradiation, adoptive transfer of pmel-1 T cells plus IL-2 therapy without vaccination can successfully treat established, palpable B16 tumors (56).

IL-2 was a critical component of regimens that also included adoptive transfer of pmel-1 transgenic $gp100_{28–33}$–specific T cells and vaccination (14, 24, 55). Interestingly, IL-2 did not substantially increase the number of $gp100_{28–33}$–specific $CD8^+$ T cells, but it did greatly increase the activation status of $gp100_{28–33}$–specific $CD8^+$ T cells at tumor sites (14). Nearly identical antitumor activity was observed when either IL-15 or IL-2 was administered in combination with adoptive transfer of pmel-1 cells and vaccination (55).

Adoptive transfer of transgenic $gp100_{28–33}$–specific $CD8^+$ T cells combined with vaccination and administration of cytokines, such as IL-2 or IL-15, is the most effective strategy for treating established B16. Obviously, the adoptive T-cell transfer component of this strategy is difficult to translate to human clinical trials because syngeneic, transgenic tumor antigen–specific $CD8^+$ T cells are not available for each patient with cancer. Obtaining large numbers of tumor-specific T cells by *in vitro* culture is very difficult under any circumstances and has only reliably been achieved for melanoma (57, 58). Moreover, the *in vivo* effectiveness of T cells might be decreased by the activation and differentiation that takes place during *in vitro* culture (54). One way to obtain large numbers of tumor antigen–specific T cells for adoptive transfer might be to introduce tumor antigen–specific TCR genes into the T cells of cancer patients. This approach has already been established in mice (59) and has been used in a limited number of human patients (60).

Conclusions

We have conducted a review of the many vaccination regimens that have been used to treat the murine B16 melanoma because we believe that the effectiveness of immune therapies against B16 might be a reasonable predictor of the effectiveness of immune therapies against human tumors. The effectiveness of vaccine regimens that have been tested against established human cancers in clinical trials has been very limited (1). Similarly, it has never been reported that any class of vaccine alone can consistently eradicate established, palpable B16. Immunosuppression followed by adoptive transfer of tumor-reactive T cells plus administration of high-dose IL-2 is an effective therapy for melanoma in humans (57, 58). The most effective therapy for established B16 tumors is adoptive transfer of tumor antigen–specific T cells combined with vaccination and IL-2 (14). This therapy is enhanced by immunosuppression prior to adoptive transfer of T cells (61). One general concept that can be taken away from our review is that prophylaxis against tumor implantation can be achieved by many types of vaccines (8, 13, 18, 20, 21, 26, 33), but eradication of established, palpable B16 tumors can only be consistently accomplished by the combination of adoptive transfer of transgenic tumor antigen–specific $CD8^+$ T cells combined with vaccination and IL-2 (14). Future murine immunotherapy experiments should attempt to develop novel therapies that can eradicate established malignancies. Development of novel immune therapies

capable of eradicating established, palpable B16 without using transgenic T cells would be an important advance.

Different effector cells were activated by the various vaccination regimens that generated antitumor immunity against B16. Antitumor immunity generated by successful vaccination regimens most commonly depended on $CD8^+$ T cells. $CD8^+$ T-cell–dependent antitumor immunity was demonstrated with recombinant viral vaccines (12), DNA vaccines (18), peptide+CpG in IFA vaccines (38), and a therapeutic regimen of vaccination with irradiated GM-CSF–transduced B16 cells plus CTLA-4 blockade (32). $CD4^+$ T cells were critical for a recombinant viral vaccine to mediate prophylaxis against a B16 challenge (6). $CD4^+$ T cells were also critical for DNA vaccines to mediate prophylaxis against a B16 challenge (18, 20). $CD4^+$ T cells were involved in protection of mice against a B16 challenge after vaccination with irradiated GM-CSF–transduced B16 cells (33). Natural killer cells were important for DNA vaccines (20) and tumor antigen–transduced DCs (29) to mediate prophylaxis against a B16 challenge. Previously injected B16 was rejected after vaccination with GM-CSF–transduced B16 and CTLA-4 blockade by a mechanism that depended on natural killer cells as well as $CD8^+$ T cells (32).

Three factors were repeatedly associated with effective treatment of B16 by vaccination. First, most regimens that were effective at treating B16 elicited large numbers of tumor antigen–specific T cells (10, 14, 31, 38, 39). Second, several effective regimens used a foreign antigen or the xenogeneic form of a self-antigen for vaccination (8, 13, 14, 18, 20, 25). Third, IL-2 was able to enhance the efficacy of multiple therapeutic regimens (14, 24, 25, 27, 38, 39). Design of future anticancer vaccination regimens should take into account these three factors that were associated with effective treatment of B16.

In a clinical trial of adoptive transfer of tumor-specific T cells, objective remissions were induced in 51% of melanoma patients (57, 58). In these patients, up to 70% of peripheral blood $CD8^+$ T cells were specific for melanoma-associated antigens (57). In a murine model, a clear correlation has been shown between the magnitude of tumor antigen–specific T-cell responses elicited by vaccination and the antitumor efficacy of vaccination (62). Consistent with this work, some of the regimens that were most effective at treating B16 elicited large numbers of tumor antigen–specific T cells, as measured by *ex vivo* tetramer or intracellular cytokine staining analyses (10, 14, 31, 38). Greater than 1% of total $CD8^+$ T cells were TRP-$2_{180–188}$ specific following vaccination with B16–GM-CSF plus CTLA-4 blockade (10, 31). Vaccination of mice with TRP-$2_{180–188}$+CpG combined with administration of systemic IL-2 generated TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses that made up 18% of total $CD8^+$ T cells (38). After adoptive transfer of transgenic $gp100_{28–33}$–specific $CD8^+$ T cells plus vaccination and administration of IL-2, greater than 50% of peripheral blood $CD8^+$ T cells were $gp100_{28–33}$ specific (14). Due to the association of large numbers of tumor antigen–specific $CD8^+$ T cells and effective tumor therapy, future vaccination regimens should be designed to maximize the number of these T cells. One way to generate large numbers of epitope-specific T cells is to include the combination of CpGs and IL-2 in vaccination regimens (38, 39, 45). Adoptive transfer is another strategy that has promise to achieve high levels of antigen-specific T cells (14, 54, 55, 57, 58, 60).

Another factor associated with effective treatment of B16 was use of xenogeneic forms of self-antigens or true foreign antigens in vaccines (8, 13, 14, 18, 20). In several studies, vaccination with plasmid DNA or recombinant viruses encoding human TRP-2 was an effective treatment for B16, whereas vaccination with vectors encoding murine TRP-2 was not (8, 12, 18). The treatment regimen of adoptive transfer of transgenic $gp100_{28–33}$–specific $CD8^+$ T cells, vaccination with human gp100, and administration of IL-2 is the most effective immunologic therapy for established B16 (14). This regimen is an example of vaccinating mice with a xenogeneic form of a self-antigen (14). The T cells elicited by vaccination with human gp100 cross-react with murine $gp100_{28–33}$ that is expressed by B16. Vaccination of mice with the murine version of $gp100_{28–33}$ did not generate significant T-cell responses (5). A similar concept has been translated to clinical trials by vaccinating patients with peptides in which one or more amino acids of the peptide were altered so that the peptide sequence was different from the normal version. This approach has already been used to elicit larger $CD8^+$ T-cell responses than vaccination with the unaltered version of the same peptide (34).

As noted previously, high-avidity $CD8^+$ T cells have been shown to be more effective than low-avidity $CD8^+$ T cells at eradicating B16 (48). Many antigens targeted by vaccination strategies are self-antigens that are expressed by normal tissues as well as by tumors. Mechanisms of tolerance eliminate most high-avidity T cells that are specific for self-antigens (50–53) One approach to obtain high-avidity T cells that have not been subjected to self-tolerance is to vaccinate human MHC–transgenic mice with human antigens that differ between mice and humans. T cells from these mice can then be used to derive T-cell clones that are specific for the human antigen. The TCR genes of these clones could then be transferred to the T cells of cancer patients for use in adoptive T-cell therapies (60, 63, 64).

IL-2 was included in several successful treatment regimens for B16 (14, 25, 27, 38). The antitumor efficacy of DC vaccines was enhanced by IL-2 (25, 27). IL-2 synergized with CpGs to cause a dramatic increase in TRP-$2_{180–188}$–specific $CD8^+$ T-cell responses and to cause epitope-specific inhibition of B16 growth (38, 39). IL-2 was a critical component of the regimen that also included adoptive transfer of transgenic $gp100_{28–33}$–specific T cells and vaccination (14, 54).

In conclusion, many different vaccination strategies have been tested against B16. These studies show that it is possible to generate potent antitumor immune responses against a poorly immunogenic tumor such as B16. In order to be effective at treating tumors, vaccination regimens should generate large T-cell responses that can be measured *ex vivo* (62). The avidity of antitumor T cells is a critical factor (48). The most effective vaccination regimens at treating established B16 include adoptive transfer of tumor antigen–specific T cells, vaccination, and cytokines such as IL-2 or IL-15 (14, 24, 54, 55).

1. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 10:909–915, 2004.
2. Bloom MB, Perry-Lalley D, Robbins PF, Li Y, el-Gamil M, Rosenberg SA, Yang JC. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma. J Exp Med 185:453–459, 1997.
3. Peter I, Mezzacasa A, LeDonne P, Dummer R, Hemmi S. Comparative analysis of immunocritical melanoma markers in the mouse melanoma cell lines B16, K1735 and S91-M3. Melanoma Res 11:21–30, 2001.
4. Seliger B, Wollscheid U, Momburg F, Blankenstein T, Huber C. Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. Cancer Res 61:1095–1099, 2001.
5. Overwijk WW, Tsung A, Irvine KR, Parkhurst MR, Goletz TJ, Tsung K, Carroll MW, Liu C, Moss B, Rosenberg SA, Restifo NP. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. J Exp Med 188:277–286, 1998.
6. Overwijk WW, Lee DS, Surman DR, Irvine KR, Touloukian CE, Chan CC, Carroll MW, Moss B, Rosenberg SA, Restifo NP. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. Proc Natl Acad Sci U S A 96:2982–2987, 1999.
7. Murali-Krishna K, Altman JD, Suresh M, Sourdive DJ, Zajac AJ, Miller JD, Slansky J, Ahmed R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity 8:177–187, 1998.
8. Steitz J, Bruck J, Steinbrink K, Enk A, Knop J, Tuting T. Genetic immunization of mice with human tyrosinase-related protein 2: implications for the immunotherapy of melanoma. Int J Cancer 86: 89–94, 2000.
9. Davila E, Kennedy R, Celis E. Generation of antitumor immunity by cytotoxic T lymphocyte epitope peptide vaccination, CpG-oligodeoxynucleotide adjuvant, and CTLA-4 blockade. Cancer Res 63:3281–3288, 2003.
10. van Elsas A, Sutmuller RP, Hurwitz AA, Ziskin J, Villasenor J, Medema JP, Overwijk WW, Restifo NP, Melief CJ, Offringa R, Allison JP. Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. J Exp Med 194:481–489, 2001.
11. Parkhurst MR, Fitzgerald EB, Southwood S, Sette A, Rosenberg SA, Kawakami Y. Identification of a shared HLA-A*0201-restricted T-cell epitope from the melanoma antigen tyrosinase-related protein 2 (TRP2). Cancer Res 58:4895–4901, 1998.
12. De Palma R, Marigo I, Del Galdo F, De Santo C, Serafini P, Cingarlini S, Tuting T, Lenz J, Basso G, Milan G, Zanovello P, Bronte V. Therapeutic effectiveness of recombinant cancer vaccines is associated with a prevalent T-cell receptor alpha usage by melanoma-specific CD8+ T lymphocytes. Cancer Res 64:8068–8076, 2004.
13. Steitz J, Bruck J, Gambotto A, Knop J, Tuting T. Genetic immunization with a melanocytic self-antigen linked to foreign helper sequences breaks tolerance and induces autoimmunity and tumor immunity. Gene Ther 9:208–213, 2002.
14. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, Dellemijn TA, Antony PA, Spiess PJ, Palmer DC, Heimann DM, Klebanoff CA, Yu ZY, Hwang LN, Feigenbaum L, Kruisbeek AM, Rosenberg SA, Restifo NP. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. J Exp Med 198:569–580, 2003.
15. Rosenberg SA, Zhai Y, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Seipp CA, Einhorn JH, Roberts B, White DE. Immunizing patients with metastatic melanoma using recombinant adenoviruses encoding MART-1 or gp100 melanoma antigens. J Natl Cancer Inst 90:1894–1900, 1998.
16. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Topalian SL, Sherry RM, Restifo NP, Wunderlich JR, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, Gritz L, Panicali DL, White DE. Recombinant fowlpox viruses encoding the anchor-modified gp100 melanoma antigen can generate antitumor immune responses in patients with metastatic melanoma. Clin Cancer Res 9:2973–2980, 2003.
17. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA. DNA vaccines. Annu Rev Immunol 15:617–648, 1997.
18. Bowne WB, Srinivasan R, Wolchok JD, Hawkins WG, Blachere NE, Dyall R, Lewis JJ, Houghton AN. Coupling and uncoupling of tumor immunity and autoimmunity. J Exp Med 190:1717–1722, 1999.
19. Tuting T, Gambotto A, DeLeo A, Lotze MT, Robbins PD, Storkus WJ. Induction of tumor antigen-specific immunity using plasmid DNA immunization in mice. Cancer Gene Ther 6:73–80, 1999.
20. Weber LW, Bowne WB, Wolchok JD, Srinivasan R, Qin J, Moroi Y, Clynes R, Song P, Lewis JJ, Houghton AN. Tumor immunity and autoimmunity induced by immunization with homologous DNA. J Clin Invest 102:1258–1264, 1998.
21. Schreurs MW, Eggert AA, de Boer AJ, Vissers JL, van Hall T, Offringa R, Figdor CG, Adema GJ. Dendritic cells break tolerance and induce protective immunity against a melanocyte differentiation antigen in an autologous melanoma model. Cancer Res 60:6995–7001, 2000.
22. Rosenberg SA, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, Restifo NP, Haworth LR, Seipp CA, Freezer LJ, Morton KE, Mavroukakis SA, White DE. Inability to immunize patients with metastatic melanoma using plasmid DNA encoding the gp100 melanoma-melanocyte antigen. Hum Gene Ther 14:709–714, 2003.
23. Bellone M, Cantarella D, Castiglioni P, Crosti MC, Ronchetti A, Moro M, Garancini MP, Casorati G, Dellabona P. Relevance of the tumor antigen in the validation of three vaccination strategies for melanoma. J Immunol 165:2651–2656, 2000.
24. Lou Y, Wang G, Lizee G, Kim GJ, Finkelstein SE, Feng C, Restifo NP, Hwu P. Dendritic cells strongly boost the antitumor activity of adoptively transferred T cells in vivo. Cancer Res 64:6783–6790, 2004.
25. Shimizu K, Thomas EK, Giedlin M, Mule JJ. Enhancement of tumor lysate- and peptide-pulsed dendritic cell-based vaccines by the addition of foreign helper protein. Cancer Res 61:2618–2624, 2001.
26. Ribas A, Butterfield LH, Hu B, Dissette VB, Chen AY, Koh A, Amarnani SN, Glaspy JA, McBride WH, Economou JS. Generation of T-cell immunity to a murine melanoma using MART-1-engineered dendritic cells. J Immunother 23:59–66, 2000.
27. Eggert AO, Becker JC, Ammon M, McLellan AD, Renner G, Merkel A, Brocker E-B, Kampgen E. Specific peptide-mediated immunity against established melanoma tumors with dendritic cells requires IL-2 and fetal calf serum-free cell culture. European J Immunol 32:122–127, 2002.
28. Toldbod HE, Agger R, Bolund L, Hokland M. Potent influence of

bovine serum proteins in experimental dendritic cell-based vaccination protocols. Scand J Immunol 58:43–50, 2003.

29. Wargo JA, Schumacher LY, Comin-Anduix B, Dissette VB, Glaspy JA, McBride WH, Butterfield LH, Economou JS, Ribas A. Natural killer cells play a critical role in the immune response following immunization with melanoma-antigen-engineered dendritic cells. Cancer Gene Ther 12:516–527, 2005.
30. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci U S A 90:3539–3543, 1993.
31. Sutmuller RP, van Duivenvoorde LM, van Elsas A, Schumacher TN, Wildenberg ME, Allison JP, Toes RE, Offringa R, Melief CJ. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J Exp Med 194:823–832, 2001.
32. van Elsas A, Hurwitz AA, Allison JP. Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. J Exp Med 190:355–366, 1999.
33. Hung K, Hayashi R, Lafond-Walker A, Lowenstein C, Pardoll D, Levitsky H. The central role of CD4(+) T cells in the antitumor immune response. J Exp Med 188:2357–2368, 1998.
34. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dudley ME, Schwarz SL, Spiess PJ, Wunderlich JR, Parkhurst MR, Kawakami Y, Seipp CA, Einhorn JH, White DE. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 4:321–327, 1998.
35. Slingluff CL Jr, Petroni GR, Yamshchikov GV, Hibbitts S, Grosh WW, Chianese-Bullock KA, Bissonette EA, Barnd DL, Deacon DH, Patterson JW, Parekh J, Neese PY, Woodson EM, Wiernasz CJ, Merrill P. Immunologic and clinical outcomes of vaccination with a multiepitope melanoma peptide vaccine plus low-dose interleukin-2 administered either concurrently or on a delayed schedule. J Clin Oncol 22:4474–4485, 2004.
36. Weber J, Sondak VK, Scotland R, Phillip R, Wang F, Rubio V, Stuge TB, Groshen SG, Gee C, Jeffery GG, Sian S, Lee PP. Granulocyte-macrophage-colony-stimulating factor added to a multipeptide vaccine for resected Stage II melanoma. Cancer 97:186–200, 2003.
37. Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, Royal RE, Kammula U, Restifo NP, Hughes MS, Schwartzentruber D, Berman DM, Schwarz SL, Ngo LT, Mavroukakis SA, White DE, Steinberg SM. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol 175:6169–6176, 2005.
38. Kochenderfer JN, Chien CD, Simpson JL, Gress RE. Synergism between CpG-containing oligodeoxynucleotides and IL-2 causes dramatic enhancement of vaccine-elicited CD8+ T cell responses. J Immunol 177:8860–8873, 2006.
39. Kochenderfer JN, Chien CD, Simpson JL, Gress RE. Maximizing CD8+ T cell responses elicited by peptide vaccines containing CpG oligodeoxynucleotides. Clin Immunol 124:119–130, 2007.
40. Miconnet I, Koenig S, Speiser D, Krieg A, Guillaume P, Cerottini JC, Romero P. CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. J Immunol 168:1212–1218, 2002.
41. Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 20:709–760, 2002.
42. Krieg AM. Development of TLR9 agonists for cancer therapy. J Clin Invest 117:1184–1194, 2007.
43. Ballas ZK, Krieg AM, Warren T, Rasmussen W, Davis HL, Waldschmidt M, Weiner GJ. Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. J Immunol 167:4878–4886, 2001.
44. Speiser DE, Lienard D, Rufer N, Rubio-Godoy V, Rimoldi D, Lejeune F, Krieg AM, Cerottini JC, Romero P. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest 115:739–746, 2005.
45. Kochenderfer JN, Simpson JL, Chien CD, Gress RE. Vaccination regimens incorporating CpG-containing oligodeoxynucleotides and IL-2 generate antigen-specific anti-tumor immunity from T cell populations undergoing homeostatic peripheral expansion after BMT. Blood 110:450–460, 2007.
46. Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M, Lee PP. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 9:1377–1382, 2003.
47. Bullock TNJ, Mullins DW, Engelhard VH. Antigen density presented by dendritic cells in vivo differentially affects the number and avidity of primary, memory, and recall CD8+ T cells. J Immunol 170:1822–1829, 2003.
48. Zeh HJ, 3rd, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. J Immunol 162:989–994, 1999.
49. Lyman MA, Nugent CT, Marquardt KL, Biggs JA, Pamer EG, Sherman LA. The fate of low affinity tumor-specific CD8+ T cells in tumor-bearing mice. J Immunol 174:2563–2572, 2005.
50. Allen PM. Peptides in positive and negative selection: a delicate balance. [comment]. Cell 76:593–596, 1994.
51. De Visser KE, Schumacher TNM, Kruisbeek AM. CD8+ T cell tolerance and cancer immunotherapy. J Immunother 26:1–11, 2003.
52. Theobald M, Biggs J, Hernandez J, Lustgarten J, Labadie C, Sherman LA. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. J Exp Med 185:833–841, 1997.
53. Hernandez J, Lee PP, Davis MM, Sherman LA. The use of HLA A2.1/p53 peptide tetramers to visualize the impact of self tolerance on the TCR repertoire. J Immunol 164:596–602, 2000.
54. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest 115:1616–1626, 2005.
55. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. Proc Natl Acad Sci U S A 101:1969–1974, 2004.
56. Wrzesinski C, Paulos CM, Gattinoni L, Palmer DC, Kaiser A, Yu Z, Rosenberg SA, Restifo NP. Hematopoietic stem cells promote the expansion and function of adoptively transferred antitumor CD8 T cells. J Clin Invest 117:492–501, 2007.
57. Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, Topalian SL, Sherry R, Restifo NP, Hubicki AM, Robinson MR, Raffeld M, Duray P, Seipp CA, Rogers-Freezer L, Morton KE, Mavroukakis SA, White DE, Rosenberg SA. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. Science 298:850–854, 2002.
58. Dudley ME, Wunderlich JR, Yang JC, Sherry RM, Topalian SL, Restifo NP, Royal RE, Kammula U, White DE, Mavroukakis SA, Rogers LJ, Gracia GJ, Jones SA, Mangiameli DP, Pelletier MM, Gea-Banacloche J, Robinson MR, Berman DM, Filie AC, Abati A, Rosenberg SA. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of

patients with refractory metastatic melanoma. J Clin Oncol 23:2346–2357, 2005.

59. Kessels HW, Wolkers MC, van den Boom MD, van der Valk MA, Schumacher TN. Immunotherapy through TCR gene transfer. Nat Immunol 2:957–961, 2001.
60. Morgan RA, Dudley ME, Wunderlich JR, Hughes MS, Yang JC, Sherry RM, Royal RE, Topalian SL, Kammula US, Restifo NP, Zheng Z, Nahvi A, de Vries CR, Rogers-Freezer LJ, Mavroukakis SA, Rosenberg SA. Cancer regression in patients after transfer of genetically engineered lymphocytes. Science 314:126–129, 2006.
61. Gattinoni L, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, Restifo NP. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. J Exp Med 202:907–912, 2005.
62. Perez-Diez A, Spiess PJ, Restifo NP, Matzinger P, Marincola FM. Intensity of the vaccine-elicited immune response determines tumor clearance. J Immunol 168:338–347, 2002.
63. Cohen CJ, Zheng Z, Bray R, Zhao Y, Sherman LA, Rosenberg SA, Morgan RA. Recognition of fresh human tumor by human peripheral blood lymphocytes transduced with a bicistronic retroviral vector encoding a murine anti-p53 TCR. J Immunol 175:5799–5808, 2005.
64. Schumacher TNM. T-cell-receptor gene therapy. Nat Rev Immunol 2: 512–519, 2002.