Vaccination with a recombinant vaccinia virus encoding a “self” antigen induces autoimmune vitiligo and tumor cell destruction in mice: Requirement for CD4+ T lymphocytes

WILLEM W. OVERWIJK*†, DAVID S. LEE*†, DEBORAH R. SURMAN*, KARI R. IRVINE*, CHRISTOPHER E. TOULOUKIAN*, CHI-CHAO CHAN‡, MILES W. CARROLL§, BERNARD MOSS‡, STEVEN A. ROSENBERG*, AND NICHOLAS P. RESTIFO*¶

*Laboratory of Immunology, National Eye Institute, and ‡Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

It has long been known that elements of the cellular immune response, and CD4+ T lymphocytes in particular, are capable of specifically recognizing and destroying tumor cells. Classical studies showed that mice immunized with irradiated methylcholanthrene-induced sarcoma cells were fully protected against a subsequent challenge with that same tumor, but not against others (1). This protection depended on CD8+ T lymphocytes, whereas CD4+ T lymphocytes often played little, if any, role. Furthermore, adoptive transfer of pure populations of CD8+ T lymphocytes mediate tumor regression in mice (2–4). Thus, CD8+ T lymphocytes have been the focus of recent efforts toward development of therapeutic anticancer vaccines (2, 5–7).

Recently, molecular targets of tumor-specific CD8+ T lymphocytes have been identified in human and mouse systems (4, 5, 8, 9). One group of antigens consists of nonmutated “self” melanocyte differentiation antigens such as gp100/pmel-17, MART-1/Melan-A, tyrosinase, and tyrosinase-related proteins (TRP) 1 and TRP-2. These antigens are expressed by both normal and malignant melanocytes (8). A pitfall in attempts to target these antigens with cancer vaccines may be central and peripheral tolerance to self-antigens, which may not be adequately modeled in many mouse tumor models using “foreign” antigens such as ovalbumin, viral proteins, or xenogeneic forms of target antigens. Self-reactive T lymphocytes can be physically or functionally deleted in the thymus or in the periphery, leaving behind only limited numbers of functionally impaired T lymphocytes (10, 11).

It is nevertheless possible to mount a potent immune response to self-antigens as evidenced in patients afflicted with autoimmune disease, as well as in animal models, where autoantigens can be targeted by immune responses powerful enough to destroy thyroid cells, pancreatic beta cells, or myelin sheaths. In patients afflicted with autoimmune vitiligo, affected skin areas are progressively depleted of melanocytes, leading to a total loss of pigment. Upon treatment of melanoma patients with interleukin (IL) 2, approximately 20% of responding melanoma patients, but none of responding renal cancer patients, developed vitiligo (12). The relationship, if any, between vitiligo and tumor regression has not been elucidated, but it is interesting that IL-2 is one of the prime cytokines secreted by CD4+ T lymphocytes. We hypothesized that deliberate induction of self-reactivity might result in antitumor effects. To test this hypothesis, we attempted to induce autoimmune vitiligo by using murine homologues of five known human melanocyte differentiation antigens and to determine the role of CD8+ and CD4+ T lymphocytes in autoimmune disease and antitumor effects.

MATERIALS AND METHODS

Animals and Cell Lines. Six- to 10-week-old female C57BL/6n (H-2b) mice were obtained from Frederick Cancer Research Center (Frederick, MD). β2-microglobulin (β2m) knockout mice, major histocompatibility complex (MHC) class II knockout mice, and perforin knockout mice, severely deficient in CD8+ T lymphocytes, CD4+ lymphocytes, and both CD8+ T lymphocytes and Natural Killer function, respectively, were obtained from Taconic Farms. All mice were maintained in a barrier facility. The spontaneous murine melanoma B16 expresses gp100, MART-1, tyrosinase, TRP-1, and TRP-2 as shown by fluorescence-activated cell sorting and Western blotting (data not shown). All tumor lines were maintained in CM [RPMI medium 1640 with 10% heat-inactivated fetal bovine serum (Biofluids, Rockville, MD), 0.03% l-glutamine, 100 μg/ml of streptomycin, 100 μg/ml of

Abbreviations: β2m, β2-microglobulin; IL, interleukin; MHC, major histocompatibility complex; TRP, tyrosinase-related protein; rVV, recombinant vaccinia virus.

†W.W.O. and D.S.L. contributed equally to this work.

‡To whom reprint requests should be addressed: National Cancer Institute, Building 10, Room 2B42, Bethesda, MD 20892-1502. e-mail: restifo@nih.gov.

PNAS is available online at www.pnas.org.
penicillin and 50 μg/ml of gentamicin sulfate (National Institutes of Health Media Center).

**Recombinant Viruses and Plasmid DNA.** All recombinant vaccinia viruses (rVV) used in this study were generated and purified as described (4, 13). rVVmp100, rVVmMART-1, rVVmTyr, rVVmTRP-1, and rVVmTRP-2 were based on the plasmid pSC65 in which the strong synthetic early/prolate promoter pSE/L drives expression of the antigen and the weaker early/late pSE/L drives expression of the *Escherichia coli* LacZ gene (14, 20). The baculovirus encoding mTRP-1 was a kind gift of Alan N. Houghton, Memorial Sloan Kettering Center, New York (15). Cloning of the genes for mpg100 and mTRP-1/Melan-A and TRP-2 has been described (16, 17). Expression of plasmid DNA and rVV was confirmed by immunostaining and Western blotting analysis of transfected and infected cells by using antisera kindly provided by V. Hearing, National Cancer Institute, Bethesda, MD (18). Interestingly, monkey kidney cells infected with rVV encoding mTyr, rate-limiting enzyme in the synthesis of eumelanin, turned intensely black within 48 hr. Recombinant plasmid DNA delivery by gene gun was described (19).

**Tumor Protection Assay.** Mice were vaccinated i.v. with 1–2 × 10⁷ plaque-forming units of rVVmTRP-1, or control virus rVVlacZ, and boosted 3 weeks later. Three to 5 weeks after the last vaccination, mice were injected s.c. on the ventral side with 1 × 10⁸ B16 tumor cells in 100 μl of PBS. Starting on day 7, the product of perpendicular tumor diameters was determined every other day until tumors became larger than 1 cm². All experiments were performed at least twice in a blinded, randomized fashion.

**Western Blotting, Dot Blotting, and Histological Analysis.** For Western blotting, cell lysates were prepared by lysing indicated cell lines in PBS with 0.02% Triton X-100 and 1% aprotinin. Sample and SDS-containing loading buffer (1:1) were mixed, boiled for 2 min, and applied on 4–20% Tris-glycine SDS/PAGE gel (NOVEX, San Diego, CA). Samples were run and blotted onto poly(vinylidene difluoride) membrane (NOVEX) and blocked in 5% powdered fat-free milk in PBS. Washes between primary antibody and horseradish peroxidase-labeled secondary antibody (Amersham Pharma) were done with 1% BSA in PBS before staining with diaminobenzidine substrate (Sigma), or incubation with chemiluminescence reagent (Western blotting Chemiluminescence Reagent Plus, NEN). For dot blotting, indicated 25-mer peptides were solubilized in 10% dimethyl sulfoxide in PBS, and 10 μg of peptide was applied directly on a nitrocellulose membrane and dried 10 min. For histology, tissues were fixed in 10% buffered formaldehyde and embedded in methacrylate, and 4-μm sections were stained with hematoxylin and eosin.

**ELISA Testing of Mouse Sera.** B16 melanoma cells were washed and resuspended at 2 × 10⁶ cells/ml in PBS (Biofluids) and then lysed by three cycles of freeze and thaw. B16 lysate at 1 × 10⁴ cell equivalents (50 μl) was plated into wells of poly(vinylidene chloride) microtiter plates (Dynex Technologies, Chantilly, VA) and dried overnight at 37°C. Wells were blocked with 100 μl of 5% BSA (GIBCO/BRL) in PBS (Biofluids) for 1 hr at 37°C. Fifty microliters of 1:2,500 experimental mouse sera in PBS with 1% BSA was added to each well and incubated for 2 hr at 37°C. Goat anti-mouse IgG₂b horseradish peroxidase conjugate was used at a dilution of 1:4,000 (Boehringer Mannheim). Five milligrams of OPD (o-phenylenediamine dihydrochloride, Sigma) and 50 μl of 3% hydrogen peroxide (Sigma) were added to 10 ml of the substrate buffer [5.1 g of citric acid (Sigma)] with 13.8 g of sodium dibasic heptahydrate (Malrinckrodt) in 1,000 ml of dH₂O. Per well, 50 μl of this substrate was added, and the colorimetric reaction was started with 50 μl of 4 M H₂SO₄ after 10 min. The wells were read at 492 nm in a Titertek Multiscan MCC/340 plate scanner.

**RESULTS**

**Immunization with rVVmTRP-1 Induces Vitiligo.** To determine the ability of melanocyte differentiation antigens to induce a self-specific immune response, we constructed rVV encoding each of five murine homologues of known human antigens recognized by T lymphocytes: gp100/pmel17, MART-1/Melan-A, tyrosinase, TRP-1/gp75, and TRP-2. Because these antigens all are expressed by normal melanocytes, we monitored vaccinated animals for autoimmune symptoms, particularly pigment abnormalities. Normal C57BL/6 mice immunized with rVV encoding mpg100, mMART-1, mTyr, or mTRP-2 did not show any evidence of depigmentation (0/15 mice in each case). Mice vaccinated with rVV encoding mTRP-1 developed profound skin depigmentation within 3 weeks after their second immunization. (168/206 mice). There was no vitiligo, however, in mice vaccinated with plasmid DNA encoding mTRP-1 (0/15 mice), or any of the other four melanocyte differentiation antigens (0/10 mice in each case), even after repeated immunizations.

Mice vaccinated with rVVmTRP-1 typically exhibited a bilateral loss of pigment, the site and pattern of which differed from mouse to mouse (Fig. 1). This pattern resembled vitiligo that occurred in some melanoma patients that responded to IL-2 treatment (12) (Fig. 1). Histopathological examination of depigmented skin of mice with vaccination-induced coat color changes revealed complete absence of pigment in hair follicles (Fig. 2B and D) as compared with skin from mice vaccinated with control rVVlacZ (Fig. 2A and C). In mice with early signs of vitiligo, depigmented skin areas bordered areas with no apparent signs of pigment loss. Interestingly, mice with pronounced skin depigmentation revealed no inflammation or loss of pigment in melanocyte-containing tissues of the eye, inner ear, and substantia nigra in the brain. This result parallels observations in vitiligo patients, where no histopathological or immunological lesions were ever detected in skin, eye, or central nervous system.
Deliberate Induction of Autoreactivity to mTRP-1 Results in Tumor Destruction. We hypothesized that the deliberate induction of self-reactivity could be useful for treatment of malignancies. To test this hypothesis, mice with vaccine-induced depigmentation were challenged s.c. with B16 melanoma. Mice vaccinated with rVVmTRP-1 were fully protected against B16 tumor challenge, with between 60% and 100% of rVVmTRP-1-immunized mice completely rejected tumor challenge. The experiment was repeated three times with similar results.

Immunization with rVVmTRP-1 Induces Class-Switched, mTRP-1-Specific Antibodies. To study the mechanism of TRP-1-specific autoimmunity, sera from mice with depigmentation after immunization with rVVmTRP-1 was assayed for the presence of TRP-1-specific antibodies by Western blotting analysis. Sera from mice immunized with rVVmTRP-1 recognized a single 75-kDa protein present in B16 melanoma lysate, but not in E22 control tumor lysate (Fig. 4A). This reactivity was identical to the reactivity of TA99, a mAb specific for mouse and human TRP-1 (ref. 21, data not shown) as well as the polyclonal rabbit sera aPEP1, raised against a synthetic TRP-1-derived peptide (18) (Fig. 4A). Sera from control-vaccinated mice did not recognize any protein in the lysates (data not shown). To identify the precise mTRP-1 peptide epitope(s) recognized by antibodies in depigmented mice, a library of 25 amino acid peptides overlapping by 10 residues was tested for recognition by sera from depigmented mice in a dot-blotting assay. Two overlapping peptides, peptide 5 (mTRP-1461–485, GRGRCVAVIADSRPHTSHYPHDGKD) and peptide 6 (mTRP-176–191, SRHYPHDGKDDEAWPLRFFNRTCO) were specifically recognized, indicating there was recognition of a linear peptide epitope contained in the shared region of peptides 5 and 6 (data not shown). However, these peptides failed to adsorb out all of the mTRP-1 reactivity from sera of depigmented mice. Moreover, mice immunized with the 15-mer consensus epitope SRHYPHDGKDDEAWPLRFFNRTCO covalently linked to keyhole limpet hemocyanin developed high-titered antibodies that were specifically reactive with the full-length mTRP-1 in lysates from B16 melanoma, but these mice never developed vitiligo and were not protected against B16 tumor challenge (data not shown), suggesting the involvement of additional antibody epitopes or immune effector mechanisms, such as T lymphocytes.

To identify whether CD4+ T lymphocytes played any role in the immune response to rVVmTRP-1, ELISA was performed on sera from immunized mice. Normal mice developed high-titered IgG2b-type antibodies that specifically reacted with mTRP-1 in B16 lysates, but mice deficient in CD4+ T-cells did not develop detectable antibodies (Fig. 4B). Similar results were obtained when sera were assayed for IgG2a-type antibodies (data not shown). This data suggested that CD4+ T lymphocytes and antibodies may play a role in rVVmTRP-1 induced melanocyte-
lymphocytes by injection of anti-CD4 mAb GK1.5 and vaccinated with rVVmTRP-1 did not develop vitiligo (0/26 mice) and were not protected from tumor challenge, whereas 60% (6/10 mice) of control mice demonstrated depigmentation and rejected B16 challenge.

In contrast, β2-m knockout mice, severely deficient in CD8+ T lymphocytes (23), that were vaccinated with rVVmTRP-1 still developed vitiligo (5/20), although this rate was significantly lower than observed in nontransgenic litter mates (17/20). Challenge of vaccinated β2-m knockout mice with B16 melanoma revealed robust protection against melanoma growth, indicating a surprisingly minor role, if any, for CD8+ T lymphocytes in the induction of antitumor immune reactivity after vaccination with rVVmTRP-1 (Fig. 5B). The lower rate of vitiligo may be caused by a well-characterized deficiency of β2-m knockout mice in the FcRn-receptor, of which β2-m forms an integral part (24, 25). This receptor protects IgG-type antibodies from clearance from the body, and its absence in β2-m knockout mice could explain the reduced effects of vaccination with rVVmTRP-1. To further clarify the role of CD8+ T lymphocytes, perforin knockout mice, deficient in CD8+ T lymphocyte and NK cell lytic function, were similarly vaccinated with rVVmTRP-1 and followed for depigmentation. In two independent experiments, 6/7 and 6/8 perforin knockout mice developed vitiligo, a rate comparable to nontransgenic litter mates and normal C57BL/6 mice, suggesting that perforin-dependent killing by CD8+ T-cells and Natural Killer cells was not essential for the induction of autoimmunity to mTRP-1. Together, these results strongly pointed to a pivotal role for CD4+ T lymphocytes in the induction of both the autoimmune and antitumor response against the self-antigen mTRP-1.

**DISCUSSION**

Our results indicate that the deliberate induction of autoimmune reactivities to a nonmutated tissue differentiation antigen can lead to tumor destruction. Vaccination with rVVmTRP-1 induced both autoimmunity and tumor protection in a manner that depended on both antibody and CD4+ T lymphocytes. In humans afflicted with autoimmune vitiligo, tolerance to melanocyte antigens apparently has been broken. One of a number of putative target antigens in this disease is tyrosinase, and elevated levels of class-switched, IgG-type melanoma-specific antibodies have been detected in sera from vitiligo patients (26). Several groups have postulated a role for antibodies in the pathogenesis of human vitiligo (26, 27).

Houghton and colleagues (21) observed that systemic administration of the TRP-1-specific mAb TA99 to mice resulted in antitumor effects as well as depigmentation (21). Furthermore, immunization with lysates of baculovirus-infected insect cells expressing mTRP-1, or plasmid DNA xenoinmunization with human TRP-1, resulted in the induction of antibody responses in vivo, suggesting a requirement for altered forms of the protein, because purified mTRP-1 protein did not elicit antibodies from clearance from the body, and its absence in β2-m knockout mice could explain the reduced effects of vaccination with rVVmTRP-1. To further clarify the role of CD8+ T lymphocytes, perforin knockout mice, deficient in CD8+ T lymphocyte and NK cell lytic function, were similarly vaccinated with rVVmTRP-1 and followed for depigmentation. In two independent experiments, 6/7 and 6/8 perforin knockout mice developed vitiligo, a rate comparable to nontransgenic litter mates and normal C57BL/6 mice, suggesting that perforin-dependent killing by CD8+ T-cells and Natural Killer cells was not essential for the induction of autoimmunity to mTRP-1. Together, these results strongly pointed to a pivotal role for CD4+ T lymphocytes in the induction of both the autoimmune and antitumor response against the self-antigen mTRP-1.

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CD4 T lymphocytes can drastically influence the initiation, maintenance, and termination of immune responses through secretion of cytokines and expression of surface molecules such as CD40 ligand. Collectively, these signals can increase antigen-presenting cell proliferation, chemotaxis, and antigen processing, as well as local MHC class I and II expression, expression of costimulatory molecules such as CD80/B7-1, and secretion of IL-12 (29, 30). In addition, CD4+ T lymphocytes can secrete specific patterns of cytokines that "steer" the immune response to a predominantly cellular, type-1 response (including interferon γ) or mainly humoral, type-2 response (including IL-4, IL-5, and perhaps IL-10) (31, 32). Once the immune response subsides, CD4+ T lymphocytes can play a determining role in the induction and maintenance of immunological memory (33–35).

The ability of CD4+ T lymphocytes to steer and amplify immune responses may explain their requirement in immunity against the self tumor antigen mTRP-1, to which some degree of immunological tolerance is likely to exist. Different groups recently have shown that immune responses to less immunogenic proteins tend to display a more pronounced dependency on "help" from CD4+ T lymphocytes (11, 36–39). In addition, classic mouse models of autoimmunity, such as experimental allergic encephalitis and systemic lupus erythematosus, typically show a strong dependence on CD4+ T lymphocytes, which can be transferred to induce disease in healthy animals (40–42). CD4+ T cells also can play a regulatory role by suppressing disease in afflicted recipients (42–45). Specific tolerization of autoreactive CD4+ T lymphocytes can completely abrogate autoimmune disease (10, 46, 47), further highlighting the prominent role for CD4+ T lymphocytes in the immunity to self-antigens.

With the cloning of a host of tumor antigens that are self, the role of CD4+ T lymphocytes in the antitumor immune response induced by antigen-specific cancer vaccines needs to be more clearly defined. Mice vaccinated with the self-antigen mTRP-1 develop autoimmune vitiligo and reject B16 melanoma expressing mTRP-1 with both phenomena revealing an absolute dependency on CD4+ T lymphocytes. However, the specificity of these CD4+ T lymphocytes remains unclear. We have been unsuccessful in isolating mTRP-1-specific CD4+ T lymphocytes by using cytokine-release and proliferation assays. Furthermore, plasmid DNA encoding mTRP-1 was not successful at inducing vitiligo. It therefore is possible that the CD4+ T lymphocytes are specific for vaccinia virus, especially if there is some physical association of TRP-1 with the vaccinia-derived helper epitope. Alternatively, the help could come from vaccinia-specific bystander CD4+ T cells that provide help locally. In either case, the helper functions could cause B lymphocytes to produce high-titered, class-switched mTRP-1-specific antibodies that are then solely responsible for tumor protection. Alternatively, mTRP-1-specific CD4+ T lymphocytes may recognize mTRP-1 released by the tumor and processed and presented by resident antigen-presenting cells.

When developing cancer vaccines using self-antigens, autoimmune destruction of normal cells expressing these antigens could be useful for cancers arising from nonessential organs, such as prostate, breast, thyroid, testis, or ovary. The autoimmune consequences of treatment targeting mTRP-1 caused vitiligo, although the mice had a normal life span. The first cohort of mice with vitiligo have been observed for 17 months, and they appear healthy, with normal size and weight. Histologically, there was no destruction of pigmented cells in the eye or ear. Likewise, melanoma patients who developed vitiligo did not experience ocular or aural changes (12, 48). Thus, the autoimmune consequences of melanoma-specific immune responses that cross-react with normal melanocytes may be acceptable when observed in conjunction with tumor regression.
We thank Dr. A. N. Houghton and Dr. V. Hearing for generous supply of recombinant baculovirus encoding mTRP-1 and mTRP-1-specific antisera, respectively, Dr. J. W. Yewdell for reading the manuscript, Mr. P. J. Spiess for expert technical assistance, and Ms. M. Blalock for expert assistance with graphics.

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