Vaccination-Induced Autoimmune Vitiligo Is a Consequence of Secondary Trauma to the Skin

Cecilia Lane, Jaina Leitch, Xiaohua Tan, Jamishid Hadjati, Jonathan L. Bramson, and Yonghong Wan

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ABSTRACT

A major concern for cancer vaccines targeting self-tumor antigens is the risk of autoimmune sequelae. Although antigen immunization correlates with autoimmune disease in some preclinical models, the mechanisms linking antigen immunization and subsequent autoimmune pathology remain to be determined. In the current study, we demonstrated that intradermal (i.d.) immunization with a recombinant adenovirus (Ad) expressing the murine melanoma antigen tyrosinase-related protein 2 (AdmTrp-2) results in a moderate level of tumor protection against the B16F10 murine melanoma without any vitiligo. Similar immunization with an Ad encoding human Trp-2 (AdhTrp-2) resulted in 50-fold greater protective immunity and produced vitiligo in all of the mice, suggesting that the development of autoimmunity may reflect the potency of the vaccine. Interestingly, delivery of AdhTrp-2 by i.m. injection generated protective immunity comparable with that seen in mice that received the vaccine by the i.d. route, but none of the recipients in the i.m. group developed vitiligo. The cellular and humoral responses in the i.m. immunized mice were greater than in the i.d. group; therefore, the lack of vitiligo was not caused by reduced efficacy of the vaccine. These results led us to hypothesize that vaccine-induced vitiligo was associated with local inflammatory responses. Mice immunized i.m. with AdhTrp-2 did develop vitiligo when they subsequently were injected i.d. with either a control Ad vector or complete Freund’s adjuvant, suggesting that vitiligo is initiated by some form of trauma within the skin. Our data demonstrated that autoimmune pathogenesis is not an unavoidable outcome of effective cancer vaccines directed against self-tumor antigens.

INTRODUCTION

Identification of tumor-associated antigens from various human cancers and animal tumor models has promoted an increasing interest in the development of antigen-specific cancer vaccines. The major challenge faced by cancer vaccines is that many potential tumor antigens also are found on normal cells; therefore, immunization against cancer must overcome self-tolerance (1, 2). The melanoma-associated antigens are prototypic self-tumor antigens. Melanocytes and melanoma cells express many of these antigens, such as gp100, tyrosinase-related protein 1 (Trp-1), Trp-2, and MART-1/Melan-A (2, 3). Studies in a murine melanoma model have demonstrated that immune tolerance to mouse melanoma antigens can be overcome by genetic immunization using viral or nonviral vectors encoding antigen genes (4–7). Furthermore, because human melanoma-associated antigens are highly homologous with murine counterparts, genetic immunization in mice can be enhanced further by incorporating human antigens, a strategy known as xenoinmunization (3, 8–11). Although the mechanisms underlying enhanced antitumor responses by genetic vaccination-expressing xenoadenogens remain elusive, results from these studies have demonstrated that multiple components of the immune system are capable of recognizing these antigens, including CD8+ T cells, CD4+ T cells, and antibodies, which can lead to tumor eradication.

A major concern for cancer vaccines is that an efficient immune response against self-tumor antigens may increase the risk of autoimmune sequelae (12). The induction of an effective immune response against Trp-1 or Trp-2 is associated with the damage of normal melanocytes manifested as vitiligo (depigmentation of patches of skin) in some animal studies of cancer vaccination (7, 13–15). Concomitant occurrence of antitumor immunity and autoimmune disease also has been observed in other nonmelanoma models (16–18). These results suggest that autoimmune destruction is an unavoidable consequence of effective antitumor immunity (7, 19, 20). However, we and others have documented that tumor protection can be achieved in the absence of autoimmune disease (11, 21, 22). The question remains whether the uncoupling of antitumor response and autoimmune pathology is caused by the differential regulation of these two events or whether it is a reflection of a mild immune response that can clean up a small number of tumor cells without causing apparent normal tissue destruction. In the current study, we describe a vaccination model in which robust antitumor immunity can be dissociated from autoimmune vitiligo. Using recombinant adenoviruses (Ads) expressing murine Trp-2 (AdmTrp-2) or human Trp-2 (AdhTrp-2) as melanoma vaccines, we can measure quantitatively the magnitude of Trp-2-specific immune responses and readily correlate antitumor immunity with the development of autoimmune vitiligo. Results from these studies demonstrated that vitiligo does not occur unless autoreactive T cells are recruited into the skin by inflammatory stimuli, suggesting that autoimmunity is not an unavoidable consequence of antitumor vaccines.

MATERIALS AND METHODS

Animals, Viruses, and Cell Culture. Six- to 8-week-old C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). CD4+CD8–, CD8+CD4–, and B cell-deficient (B220+) mice (initially purchased from The Jackson Laboratory, Bar Harbor, ME) were bred in our pathogen-free animal facility. C57BL/6 mouse-derived melanoma B16F10 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mm l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. AdmTrp-2 and AdhTrp-2 are E1/E3-deleted Ads that express the full-length murine or human TRP-2 gene. AdLacZ is an E1/E3-deleted virus that expresses β-galactosidase. Ad vectors were propagated in 293 cells and purified on a CsCl gradient as described previously (23).

Peptides. The K b -binding peptide of chicken egg ovalbumin (OVA257–264; SIINFEKL) and the K b -binding peptide shared by mTrp-2 and hTrp-2 (Trp- 2180–184; SVYDYFFVWL) were purchased from Dalton Chemical Laboratories Inc. (Toronto, Canada). Peptides were dissolved in distilled water and stored at −20°C.

Immunization and Tumor Challenge. Mice were immunized intradermally (i.d.) or i.m. with 1 × 106 plaque-forming units of AdmTrp-2 or AdhTrp-2 in 30 μl (i.d.) or 50 μl (i.m.) of PBS. Control animals received either PBS or AdLacZ. Fourteen days later, immunized animals were challenged by s.c. injection with various numbers of B16F10 cells. Tumor size was monitored daily and measured twice a week. Immunodepletion studies were conducted using the following monoclonal antibodies: GK1.5 (anti-CD4; American Type Culture Collection, Manassas, VA) and 2.43 (anti-CD8; American Type C-
Uncoupling of Vitiligo and Tumor Protection

Development of Autoimmune Vitiligo Is Associated with Enhanced Vaccination Using hTrp-2 as a Xenoantigen. We and others have reported that the first generation of recombinant Ad can be used as an effective vector for gene-based cancer vaccination (25–28). To evaluate such a vaccination strategy in a murine melanoma model, we constructed Ad vectors expressing the mouse Trp-2 (AdmTrp-2) or human Trp-2 (AdhTrp-2) protein. Intradermal immunization of C57BL/6 mice with either AdmTrp-2 or AdhTrp-2 elicited protective immunity against challenge with B16F10 cells that express endogenous Trp-2 antigen (Fig. 1A), confirming that Ad is a potent vaccination platform. Mice immunized with control vector AdLacZ or PBS developed tumors in 14 days, demonstrating that tumor protection was Trp-2 specific (Fig. 1A).

To quantify the potency of the AdmTrp-2 and AdhTrp-2 vaccines, immunized mice were challenged by s.c. injection with 2 × 10^4 to 1 × 10^5 B16F10 cells. As summarized in Fig. 1B, immunization with AdmTrp-2 only protected mice against the lowest dose of B16F10 cells (2 × 10^4), whereas 50-fold greater protective immunity was achieved with AdhTrp-2. Interestingly, all of the AdhTrp-2-immunized animals developed localized vitiligo at the vaccination sites, whereas those immunized with AdmTrp-2 did not (Fig. 2; data not shown). These results suggest that autoimmunity is a function of the strength of the vaccine.

CD4⁺ and CD8⁺ T Cells Mediate Tumor Protection and Vitiligo Development Following Intradermal Immunization with AdhTrp-2. To determine whether the same effectors are involved in tumor protection and vitiligo, mice were immunized with AdhTrp-2 and subsequently depleted of CD4⁺ and CD8⁺ cells before tumor challenge or onset of vitiligo. Protection in mice immunized with AdhTrp-2 was abrogated only by simultaneous depletion of CD4⁺ and CD8⁺ T cells, indicating that either CD4⁺ or CD8⁺ T cells could independently mediate immune responses against mTrp-2 antigen-bearing tumor cells (Fig. 3). Similar to tumor protection, only simultaneous depletion of CD4⁺ and CD8⁺ T cells, but neither population alone, abolished the incidence of vitiligo following i.d. immunization with AdhTrp-2 (Fig. 2). These results suggest that Trp-2-specific immune responses against melanoma and normal melanocytes share the same effector pathways. Previous studies have demonstrated that CD4⁺-dependent antitumor immunity may involve antibodies specific for tumor antigen as the final effector components in mediating tumor cell eradication (7, 10, 13). Antibodies do not appear to be involved in either tumor protection or the development of vitiligo because B cell-deficient mice immunized i.d. with AdhTrp-2 were protected fully against B16F10 cells (Fig. 4) and developed vitiligo (Fig. 2).

Intramuscular Immunization with AdhTrp-2 Induces Strong Antigen-Specific Immune Responses without the Induction of Vitiligo. A previous report demonstrated that the efficacy of an Ad-expressing human gp100 against B16 melanoma was route dependent (25). In that study, i.d. immunization with Adhgp100 produced protective antitumor immunity, whereas i.m. injection did not. To determine whether the antitumor immunity and vitiligo observed...
in our experiments were route dependent, mice were immunized with AdhTrp-2 i.m. In contrast to the experiments with hgp100, mice immunized with AdhTrp-2 i.m. were protected from tumor challenge to the same extent as mice immunized i.d. (Table 1). Strikingly, however, none of the mice in the i.m. group developed vitiligo.

The lack of vitiligo in the i.m. group may reflect differences in the antitumor effectors produced by the i.d. and i.m. groups. However, antitumor immunity induced by AdhTrp-2 i.m. depended on CD4 T cells and CD8 T cells similarly to the i.d. group and was unaffected in B cell-deficient mice (data not shown). Thus, the discrepancy in vitiligo induction does not appear to reflect different effector mechanisms.

To compare further the immune responses elicited by AdhTrp-2 delivered i.m. and i.d., we measured the CD8 T-cell response to the vaccine using two functional assays: (a) in vivo cytotoxic activity and (b) direct ex vivo secretion of IFN-γ. To analyze Trp-2-specific cytotoxicity in vivo, syngeneic spleen cells were pulsed with Trp-2 peptide (Trp-2_{180-188}) and labeled with a high level of CFSE. OVA_{257-264} peptide-pulsed syngeneic spleen cells were labeled with a lower level of CFSE and used as control for antigen specificity. A 1:1 mixture of 5 × 10^5 cells of each target cell population was injected i.v. into naive or AdhTrp-2-immunized mice. After 6 h, the presence of target cell populations in spleens was analyzed by flow cytometry. As shown in Fig. 5A, target cells pulsed with Trp-2 peptide were cleared selectively from mice immunized i.m. and i.d. To enumerate directly peptide-specific CD8 CTL in vaccinated mice, freshly isolated splenocytes were restimulated briefly (6 h) with Trp-2_{180-188} and the frequency of IFN-γ-secreting CD8 T cells was measured by flow cytometry. An example of the results from the flow cytometry is provided in Fig. 5A; i.m. and i.d. immunization with AdhTrp-2 could elicit Trp-2_{180-188}-specific CD8 T cells, whereas only background IFN-γ production was observed in cultures containing OVA_{257-264}. In repeated experiments, the response elicited i.m. actually was more potent than the response produced by i.d. immunization (Fig. 5B). Although antibodies do not appear to be required for either antitumor immunity or vitiligo in this model, antibody production against Trp-2 was observed in cultures containing OVA_{257-264}.

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from mice 4 weeks after immunization and examined by Western blot analysis using protein extracts from AdhTrp-2- or AdLacZ-infected 293 cells. Similar to the induction of CD8+ CTL, i.m. immunization elicited a higher level of Trp-2-specific antibody production than i.d. injection (Fig. 5C).

**Vitiligo Can Be Induced by Inflammatory Stimuli in Mice Immunized Intramuscularly with AdhTrp-2.** Because mice immunized by i.m. injection of AdhTrp-2 exhibit antitumor immunity comparable with those immunized by i.d. injection, the absence of vitiligo in the i.m. group simply cannot be a result of inadequate immunization. Interestingly, whereas vaccine-induced autoimmune vitiligo was observed consistently in all of the animals immunized with AdhTrp-2 i.d., the vitiligo was observed only around the injection site (Fig. 2). This observation prompts us to hypothesize that vitiligo may be a consequence of inflammation in the skin following vaccination, leading to the recruitment of autoreactive T cells into the skin and subsequent vitiligo. Accordingly, we reasoned that if recruitment of autoreactive T cells into the skin was a necessary step for the induction of autoimmune vitiligo following AdhTrp-2 vaccination, we should be able to induce vitiligo in i.m. immunized mice by i.d. injection with nonspecific inflammatory stimuli. To first confirm the possibility that inflammation induced by the Ad vector was sufficient to recruit anti-Trp-2 effectors into the skin, we immunized mice with AdhTrp-2 i.m. and injected AdLacZ into the skin. As a control, mice were immunized with AdLacZ i.m. and subsequently with AdhTrp-2 i.d. Consistent with our hypothesis, all of the mice immunized with AdhTrp-2 i.m. and AdLacZ i.d. did not (Table 2). To demonstrate further that the recruitment of the autoimmune effectors to the skin can result from nonspecific inflammation, mice were immunized with either AdhTrp-2 or AdLacZ i.m. followed by i.d. administration of Complete Freund’s Adjuvant (CFA) or PBS. Again, the mice immunized with AdhTrp-2 developed vitiligo in the area where the CFA was injected, whereas those immunized with AdLacZ did not (Table 2). Furthermore, vitiligo was not seen in AdhTrp-2-immunized mice that received i.d. challenge of PBS (Table 2). Thus, these results clearly demonstrate that vitiligo in this model is the result of secondary inflammation within the target tissue and not a direct result of effective antitumor immunity.

**DISCUSSION**

A major concern for cancer vaccines is that an efficient immune response against self-tumor antigens may increase the risk of autoimmune sequelae (12). A number of researchers have suggested that the induction of autoimmune disease is an inevitable consequence of generating effective antitumor immunity (7, 19, 20). Evidence for this link comes from clinical trials in which vitiligo was correlated with clinical responses to interleukin 2 therapy in patients with metastatic melanoma (29). This connection was supported additionally by animal studies in which protective antimalanoma immunity was associated largely with vitiligo development (7, 30). However, research from our group and others has demonstrated that tumor rejection can be achieved without autoimmune consequences (11, 22, 31–33). These conflicting results clearly indicate that additional studies are required to understand the mechanisms that lead to or prevent the occurrence of autoimmune consequence following cancer vaccination.

One simple explanation for the dissociation of antitumor immunity and autoimmune destruction in cancer vaccination studies is the differential level of a target antigen expressed by the tumor (high expression) and normal tissue (low expression; Refs. 34–36). Therefore, the lack of autoimmunity in some cancer vaccine models could reflect a weak vaccine that is able to eliminate a small number of tumor cells under experimental conditions without causing pathology in normal tissues. Supporting this hypothesis, vaccination with AdhTrp-2 i.d. in our model elicited modest protective immunity against a low dose of B16F10 cells (2 × 10⁶) without causing subsequent autoimmune vitiligo. Similar observations have been obtained in our studies of gp100-based vaccines and other investigations using different antigens and vaccination platforms (11, 22, 31–33). Thus, it clearly is possible to elicit protective immunity against low-dose tumor cell challenge without subsequent autoimmune syndromes. However, protective immunity provided by immunization with AdmTrp-2 was lost when the challenge was increased to 1 × 10⁷ cells. To increase the potency of the Ad vaccine, we used hT2P-2 as the antigenic target because it has been shown previously that xenogeneic antigens are more immunogenic than the native murine antigen (2, 3, 8–11). Vaccination with AdhTrp-2 provided protection against challenge with 1 × 10⁶ tumor cells, demonstrating the increased potency of the xenogeneic as a vaccine target. More importantly, all of the mice immunized with AdhTrp-2 by either i.d. or i.m. injection of reporter adenovirus encoding human Trp-2 (AdhTrp-2) either intradermally (i.d.) or i.m. elicited potent cellular and humoral responses specific for Trp-2. A, mice were immunized with AdhTrp-2 by either i.d. or i.m. injection for 14 days. Upper panels, 5.6-carboxyfluorescein succinimidyl ester (CFSE)-labeled target cells were adoptively transferred into immunized recipient for in vivo CTL 6 h before harvesting tissues, and the number in the right corner represents the percentage of IFN-γ-producing CD8+ T cells in total CD8+ T cells following stimulation with SVY-DFFVWL – the frequency of IFN-γ-producing CD8+ T cells in total CD8+ T cells following stimulation with SINFEKL. Lower panels, splenocytes from immunized mice were restimulated immediately with SINFEKL (OVA257–264) or SVYDFFVWL (Trp-2–11001–11002) peptide for intracellular cytokine staining, and the number on the top is the frequency of CFSE-labeled CFSE+ T cells in total CD8+ T cells. CFSE+ T cells after restimulation of cells in tissue culture media was used as negative control. B, summary of in vivo CTL and intracellular staining data from four experiments. C, serum samples were collected from mice (3/group) 28 days after immunization and tested for the presence of Trp2-specific antibodies by immunostaining of cell lysates from 293 cells infected with AdhTrp-2 or AdLacZ (not shown). Each number on the top of panels represents an individual mouse sample, and this assay was repeated once with similar results.

![Fig. 5](image-url) Immunization with adenovirus encoding human tyrosinase-related protein 2 (AdhTrp-2) either intradermally (i.d.) or i.m. elicits potent cellular and humoral responses specific for Trp-2. A, mice were immunized with AdhTrp-2 by either i.d. or i.m. injection for 14 days. Upper panels, 5.6-carboxyfluorescein succinimidyl ester (CFSE)-labeled target cells were adoptively transferred into immunized recipient for in vivo CTL 6 h before harvesting tissues, and the number in the right corner represents the percent-specific lysis to naïve controls. Lower panels, splenocytes from immunized mice were restimulated immediately with SINFEKL (OVA257–264) or SVYDFFVWL (Trp-2–11001–11002) peptide for intracellular cytokine staining, and the number on the top is the frequency of IFN-γ-producing CD8+ T cells in total CD8+ T cells following stimulation with SVY-DFFVWL – the frequency of IFN-γ-producing CD8+ T cells in total CD8+ T cells following stimulation with SINFEKL. B, summary of in vivo CTL and intracellular staining data from four experiments. C, serum samples were collected from mice (3/group) 28 days after immunization and tested for the presence of Trp2-specific antibodies by immunostaining of cell lysates from 293 cells infected with AdhTrp-2 or AdLacZ (not shown). Each number on the top of panels represents an individual mouse sample, and this assay was repeated once with similar results.

Table 2: Induction of vitiligo by inflammatory stimuli in mice immunized i.m. with AdhTrp-2

<table>
<thead>
<tr>
<th>i.m.</th>
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<th>Vitiligo</th>
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<tr>
<td>AdhTrp-2</td>
<td>AdLacZ</td>
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<tr>
<td>AdhTrp-2</td>
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<td>AdLacZ</td>
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*AdhTrp-2, adenovirus encoding human Trp-2.*
mice immunized with AdhTrp-2 i.d. developed vitiligo, supporting the argument that vitiligo is a reflection of vaccine efficacy. Interestingly, however, AdhTrp-2-induced vitiligo did not occur when the vaccine was delivered i.m., although anti-Trp-2 immunity elicited in mice immunized i.m. was comparable with the response in mice immunized i.d. These results suggest that vitiligo is not an inevitable outcome of self-antigen-specific autitumor immunity.

These results appear to contradict previous reports demonstrating that antitumor immunity elicited by a Trp-1-based vaccine always was associated with vitiligo (7, 10). A possible explanation for this discrepancy is that immunity induced against two closely related autoantigens involves different mechanisms (i.e., vaccine-induced immunity against Trp-1 is mediated primarily by antibodies), whereas Trp-2-specific tumor protection depends on T cell-mediated responses (5, 10, 33). Although our studies demonstrated that antibodies against Trp-2 were induced in wild-type mice immunized with AdhTrp-2, tumor protection and autoimmune vitiligo could be achieved in B cell-deficient mice, suggesting that antibodies have a minimal role. It is likely that Trp-1-specific antibodies can readily penetrate into the skin, resulting in the damage of normal melanocytes, whereas access to the skin by Trp-2-specific autoreactive T cells is extremely limited in the absence of inflammation. The observation that i.d., but not i.m., delivery of AdhTrp-2 induces vitiligo strongly supports the notion that a local inflammatory response is required to recruit Trp-2-specific effector T cells into the skin. To evaluate directly this hypothesis, we induced inflammation in the skin of mice immunized with AdhTrp-2 i.m. Two inflammatory agents were used: (a) Ad LacZ and (b) CFA. All of the AdhTrp-2-immunized mice developed vitiligo around the site where the inflammatory agent was injected in the skin, whereas control mice that were challenged with PBS or immunized with Ad LacZ did not develop any vitiligo. Furthermore, vitiligo also occurred in AdhTrp-2-immunized (i.m.) mice when their skin was bombarded twice with DNA (no specific antigen)-coated gold particles by gene gun or when they had skin lesions resulting from fights with cage-mates (data not shown). Thus, our results with the AdhTrp-2 model strongly support the concept that vitiligo following cancer vaccination is not a necessary outcome and may be a result of damage to the skin induced by local traumatic or inflammatory stimuli.

Colella et al. reported that whereas i.v. immunization with vaccinia virus expressing human tyrosinase could elicit a CTL response that cross-reacted with murine tyrosinase peptide, such autoreactive CTLs did not cause vitiligo in immunized mice (37). However, s.c. injection with a tyrosinase-specific CTL line derived from vaccinia/human tyrosinase-immunized mice induces vitiligo in the vicinity of the injection site, supporting our hypothesis that the skin access by effector T cells is required for the subsequent destruction of normal melanocytes (37). Likewise, other studies demonstrated that immunity-induced vitiligo occurred only in the area surrounding the site of tumor inoculation or skin depilation (5, 38, 39). Consistent with our observation, vitiligo in these studies rarely is dispersed into other skin area.

Increasing evidence has pointed to a critical role of autoreactive T cells, including CD4+ and CD8+ T cells, in antitumor immunity; our results suggest that activation of either CD4+ or CD8+ T cell-dependent cellular effector pathway will not necessarily induce autoimmune consequences unless an inflammatory agent is introduced into the normal tissue. Our studies offer a new explanation for the inconsistent linkage between antitumor immunity and autoimmune pathology in preclinical models and suggest that it should be possible to design a cancer vaccine strategy that will not produce autoimmune sequelae.


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