Abstract

Eradicating malignant tumors by vaccine-elicited host immunity remains a major medical challenge. To date, correlates of immune protection remain unknown for malignant mesothelioma. In this study, we demonstrated that antigen-specific CD8+ T-cell immune response correlates with the elimination of malignant mesothelioma by a model PD-1–based DNA vaccine. Unlike the nonprotective tumor antigen WT1-based DNA vaccines, the model vaccine showed complete and long-lasting protection against lethal mesothelioma challenge in immunocompetent BALB/c mice. Furthermore, it remained highly immunogenic in tumor-bearing animals and led to therapeutic cure of preexisting mesothelioma. T-cell depletion and adoptive transfer experiments revealed that vaccine-elicited CD8+ T cells conferred to the protective efficacy in a dose–dependent way. Also, these CD8+ T cells functioned by releasing inflammatory IFNγ and TNFα in the vicinity of target cells as well as by initiating TRAIL-directed tumor cell apoptosis. Importantly, repeated DNA vaccinations, a major advantage over live-vectored vaccines with issues of preexisting immunity, achieve an active functional state, not only preventing the rise of exhausted PD-1+ and Tim-3+ CD8+ T cells but also suppressing tumor-induced myeloid-derived suppressive cells and Treg cells, with the frequency of antigen-specific CD8+ T cells inversely correlating with tumor mass. Our results provide new insights into quantitative and qualitative requirements of vaccine-elicited functional CD8+ T cells in cancer prevention and immunotherapy. Cancer Res; 74(21); 6010–21. ©2014 AACR.

Introduction

Mesothelioma is a life-threatening malignant tumor in humans (1). Significant progresses have been made with vaccines that can prevent oncavirus-associated liver and cervical cancers (2, 3). The passive administration of tumor-specific antibodies or immune cells such as cytotoxic T lymphocytes (CTL), lymphokine-activated killer cell, and dendritic cells (DC) has been clinically beneficial to patients (4–6). Despite these progresses, preventing and eradicating malignant tumor such as mesothelioma by vaccine-induced immune surveillance remains difficult even in animal model systems.

Immune surveillance prevents and eliminates transformed tumor cells (7). The activation of oncogenes induces malignant tumor while rendering them vulnerable to antitumor immune effector cells such as CTLs and CD4+ Th1 cells (8, 9). When the host immune system fails to eliminate tumor cells in time, they may hijack ineffective immune responses to foster the growth and progression of cancer (10). On the one hand, tumor cells express inhibitory ligands on their surface to dysregulate T-cell function through interaction with T-cell receptors such as programmed cell death protein 1 (PD-1), CTL-associated antigen 4 (CTLA-4), and immune regulator T cell immunoglobulin mucin 3 (Tim-3; refs. 8, 11). On the other hand, the growing tumor evades immune surveillance by sustaining immunosuppressive cells such as myeloid-derived suppressive cells (MDSC) and CD4+ CD25+Foxp3+ regulatory T lymphocytes (Treg) systematically as well as in the tumor microenvironment (12). We hypothesized that an effective cancer vaccine should harness the immune system and reinstate immune surveillance by overcoming tumor-associated immune suppression. To test this hypothesis, we sought to determine the efficacy and immune correlates of protection of conventional tumor antigen WT1 DNA vaccines and a soluble PD-1 (sPD1)-based fusion DNA vaccine, namely sPD1-p24fc, via in vivo electroporation (EP) delivery against malignant mesothelioma in immunocompetent BALB/c mice. The model xenoantigen p24 is derived from HIV capsid protein GAG (13). sPD1-p24fc/EP DNA vaccination induces uniquely high frequency of
antigen-specific CD8+ T cells with broad reactivity, long-term memory, polyfunctionality, and cytotoxicity by targeting the p24 to DCs while triggering IL12 production and antigen cross-presentation (13). Using a malignant murine mesothelioma tumor engineered to express WT1 or GAG, we demonstrate that functional antigen-specific CD8+ T cells are essential to achieve complete prevention and therapeutic cure of mesothelioma.

Materials and Methods

Cell culture
AB1 cells, purchased from European Collection of Cell Cultures, and B16F10 cells, a kind gift from Dr. Kevin Ng (Department of Surgery, HKU LKS Faculty of Medicine, Hong Kong SAR, P.R. China), were maintained in DMEM supplemented with 10% FBS and antibiotics. Medium for WT1-, GAG-, and luciferase-expressing cell lines was supplemented with 1 µg/mL puromycin (Invitrogen).

Mice
All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of The University of Hong Kong (HKU #2438-11). Five- to 8-week-old female BALB/c, C.B-17/Scid-scid (SCID), and C57BL/6 mice were maintained according to standard operational procedures at HKU Laboratory Animal Unit.

Mouse immunization and tumor challenge
Vaccination was performed as described previously (13). For tumor inoculation, BALB/c or SCID mice were injected with a lethal dose of 5 × 10^5 AB1, AB1-WT1, or AB1-GAG cells, whereas C57BL/6 mice received 5 × 10^6 B16F10-GAG cells in the right hind flank subcutaneously. Live bioluminescence images were taken weekly. Tumor size was measured using calipers [tumor volume = 1/2(length × width^2)]. Under HKU Laboratory Animal Unit guidelines, mice were sacrificed when tumor size was >15 mm.

WT1-, GAG-, and luciferase-expressing mesothelioma and melanoma cell lines
The generation of these cell lines by retroviral vector pBABE-luc-puro is depicted in Supplementary Figs. S1 and S2. 293T cells were cotransfected with pBABE-based vector and the packaging plasmid pCL-Amphotropic to generate retroviral supernatants (14). AB1 and B16F10 cells were infected with corresponding supernatants and selected with 1 µg/mL puromycin. Single clones were obtained by limiting dilution of puromycin-resistant cells and screened under the IVIS100 Imaging System, and the luciferase expression was measured within the region of interest (ROI).

Flow cytometry analysis, Elispot, and serum ELISA for assessing p24-specific immune responses
Cell surface and intracellular immunostaining, Elispot as well as ELISA were performed as previously described (13). CD8-specific tetramer against HIV-1 GAG p24 gagA-I epitope AMQMLKETI was used for assay (Beckman Coulter). Flow cytometric data analysis was performed using the FlowJo software (Tree Star, v7.6).

In vivo CD4+ and CD8+ T-cell depletion study
During prophylactic or therapeutic vaccination, BALB/c mice were injected intraperitoneally (i.p.) with 1 mg anti-CD4 mAb (YTS191.1, Bio X cell) or anti-CD8 mAb (YTS169.4, Bio X cell) one day before tumor inoculation and repeated weekly (15–17). Successful T-cell depletion was confirmed by flow cytometric analysis of peripheral blood mononuclear cells.

T-cell purification and adoptive transfer
CD4+ or CD8+ T cells were isolated using Dynabeads Untouched T Cell Kits (Invitrogen) from splenocytes of vaccinated BALB/c or C57BL/6 mice 2 weeks after the last immunization. For adoptive transfer, 1.5–2 × 10^6 CD4+ or CD8+ T cells in 100 µL PBS were injected intravenously via tail vein into each mouse that were preinoculated with tumor cells (Fig. 4F; refs. 18, 19).

T-cell cytotoxicity and TRAIL blocking assay
The LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) was used to define the cytotoxic effect of splenocytes or purified T cells as previously described (13). For the TRAIL blocking assay, activated CD8+ T cells as effector were cocultured with AB1-GAG cells as target at an effector:target (E:T) ratio of 10:1 (Fig. 4H) with or without 2 µg/mL of TRAIL blocking (clone N2B2, eBioscience), Fas ligand blocking (clone MFL4, eBioscience) antibodies or isotype control before flow cytometric analysis (20).

Cytokine production assay
A total of 2 × 10^6 splenocytes or purified CD4+ or CD8+ T cells from immunized mice were cocultured with γ-irradiated AB1-GAG cells at a 10:1 ratio of effector to tumor cell. IL2 was not added in culture medium during the first two days (18). Supernatants from day 2, 5, and 7 after coculture were quantified for cytokines using the mouse Th1/Th2/Th17/Th22 13-plex FlowCytomix Kit (eBioscience) according to the manufacturer’s instructions.

Cell-cycle analysis
A total of 5 × 10^6 AB1-GAG cells were seeded into each well of 24-well plate. Twenty-four hours later, culture medium was replaced with 500 µL of previously collected supernatants (from day 5 after coculture of CD8+ T cells and γ-irradiated AB1-GAG cells). Cells were incubated for an additional 48 hours, resuspended in 500 µL culture medium containing 1 µL of Vybrant DyeCycle Violet Stain (Invitrogen) and then incubated at 37°C with 5% CO2 for 30 minutes before analysis using FACSCalibur (BD Biosciences) and FlowJo software (Tree Star, v7.6).

Isolation of splenocytes and tumor cells
Splenocytes were isolated as previously described (13). Tumors were removed using forceps and cut into 1 mm³ pieces by surgical scissors before being treated with DMEM containing 1 ml/g collagenase IA (Sigma) and DNase I (Roche) for 2 hours on a rocking platform at 37°C. The resulting cell suspension was treated with BD Pharm Lyse Lysing Buffer and passed through a 70 µm cell strainer. To
obtain tumor-infiltrating lymphocytes (TIL), cells were resuspended in 5 mL 33% Percoll (in Hank’s Balanced Salt Solution) and centrifuged at 2,300 rpm for 20 minutes.

**Statistical analysis**

A two-tailed Student t test was performed to determine statistical significance. P < 0.05 was considered to be statistically significant. Survival of challenged mice was plotted on Kaplan–Meier survival curve and the observed difference was determined using a log-rank test (GraphPad Prism 5 software, www.graphpad.com). Data represent mean values ± SEM. Correlation analyses were performed by linear regression. EC50 value was calculated according to a four-parameter logistic function.

**Results**

**Active DNA vaccination protects mice completely against lethal mesothelioma cell challenge**

As WT1-based DNA vaccines were capable to reject implanted leukemia cells (21), we sought to determine the efficacy of two similar vaccines, TruncWT1His and Full WT1His (Supplementary Fig. S1A and S1B), in inhibiting mesothelioma growth. While wild-type AB1 cells do not express WT1, we established an AB1-WT1 mesothelioma model (Supplementary Fig. S1C–S1F). The expression of WT1 was confirmed by Western blotting and flow cytometry (Supplementary Fig. S1D and S1E). Strong correlations were observed between the AB1-WT1 cell number and luciferase signal intensity (Supplementary Fig. S1F, left; \( r^2 = 0.9941 \)) and between in vivo tumor volume and luciferase signal intensity (Supplementary Fig. S1F, right; \( r^2 = 0.9519 \)). We then immunized BALB/c mice at the dose of 100 μg DNA intramuscularly via EP three times at 3-week intervals (Fig. 1A; ref. 13). Both vaccines induced similar levels of WT1-specific IgG antibody (Fig. 1B) and IFN-γ Elispots in splenocytes (Fig. 1C) as compared with PBS controls. Subsequently, mice were challenged subcutaneously with a lethal dose of 5 × 10^3 AB1-WT1 cells 2 weeks after the last vaccination. Both vaccines failed to control tumor growth (Fig. 1D) or to prolong survival (Fig. 1E). Only weak cytotoxicity effect was observed in Full WT1-vaccinated mice at an E:T ratio of 20:1 (Fig. 1F). Conventional WT1-based DNA/EP vaccines, therefore, induced limited immune responses against malignant mesothelioma.

As sPD1-p24fc/EP DNA vaccination could elicit up to 21.2% of tetramer-positive CD8+ T cells in spleen (13), we aimed to study the correlation of effective immune responses and mesothelioma control by utilizing this model vaccine. Therefore, a xenoantigen GAG-expressing mesothelioma cell line (AB1-GAG) was generated, with equal tumorigenicity and lethality as compared with wild-type AB1 (Supplementary Fig. S2A–S2F). Using the same immunization schedule and lethal challenge dose as WT1-based vaccines (Fig. 2A; ref. 13), sPD1-p24fc/EP proved significantly more efficient than controls (p24fc/EP or PBS) at eliminating implanted AB1-GAG cells (Fig. 2B), which resulted in complete rejection 10 days after challenge and mice remained tumor-free for more than 40 days (Fig. 2C and F). These protected mice also completely rejected tumor 9 days after rechallenge and remained tumor-free for 30 days after a second rechallenge (Fig. 2D).

![Figure 1. Conventional WT1 DNA vaccines showed weak immunity and failed to protect mice from mesothelioma challenge.](image)

A, schematic representation of prophylactic study. B and C, after vaccination, anti-WT1 immune responses were assessed by ELISA for antibody (B) and cellular responses in the splenocytes by IFN-γ Elispot (C). Data shown are representative of two independent experiments with three mice per group. D and E, AB1-WT1 challenge was assessed by growth curve (D) and survival (E) in vaccinated mice \( n = 5 \); F, CTL assay on splenocytes of vaccinated mice. Data represent mean ± SEM. * P < 0.05. Two independent experiments were performed, with consistent data obtained.
weeks (Fig. 2D–F). In contrast, all control mice developed tumors and died within 30 days (Fig. 2E). Splenocytes of sPD1-p24fc/EP-immunized mice showed a strong cytotoxicity effect against AB1-GAG cells in vitro even at 0.5:1 E:T ratio (Fig. 2G). However, sPD1-p24fc/EP did not control wild-type AB1 tumor growth (Fig. 2H), suggesting that antigen-specific immune responses are essential for complete and long-lasting protection.

Therapeutic cure of mesothelioma by vaccine-elicited immune responses

So far, conventional DNA vaccines have little therapeutic efficacy against solid tumors (22, 23). To determine the therapeutic effects of sPD1-p24fc/EP, mice were first inoculated subcutaneously with the lethal dose of AB1-GAG cells followed by sPD1-p24fc/EP, p24fc/EP or sham vaccination the next day and every 2 weeks for four times (Fig. 3A). Critically, sPD1-p24fc/EP remained immunogenic with potent p24-specific CD8+ T-cell and antibody (both IgG1 and IgG2a) responses induced in tumor-bearing mice (Fig. 3B). Tetramer+ CD8+ T cells reached an average of 17.5% of total splenic CD8+ T cells (13). The first sPD1-p24fc/EP vaccination did not alter tumor growth similar to p24fc/EP or sham (Fig. 3C and E), which established approximately 10 mm in diameter. The control of these established solid tumors was observed after the second vaccination, and complete rejection of tumor was obtained after the third vaccination at day 29 in 3 of 5 mice (Fig. 3C–E). In contrast, p24fc/EP failed to show any therapeutic effect. All mice in the control groups died on day 38, whereas 100% survival was achieved by sPD1-p24fc/EP (Fig. 3D). An additional sPD1-p24fc/EP at day 43 resulted in complete rejection of solid tumor 7 days later in all mice (Fig. 3D and E), and survived more than 30 weeks with no tumor recurrence (Fig. 3D). Inoculation of AB1-GAG cells in the PBS group elicited low anti-p24 immune responses that could not prevent tumor growth (Fig. 3D and E). Hence, repeated sPD1-p24fc/EP DNA vaccination demonstrated not only potent immunogenicity but also therapeutic cure of malignant mesothelioma in tumor-bearing BALB/c mice.

![Figure 2. Complete protection of mice against lethal mesothelioma cell challenge. A, schematic representation of prophylactic study as color-coded for each group. B and C, vaccinated mice (n = 5) were assessed by tumor growth (B) and survival (C) after AB1-GAG challenge. D and E, forty days after tumor ablation, mice (n = 5) were rechallenged and measured for tumor growth (D) and survival (E). F, representative BLIs of AB1-GAG tumor in both tumor-challenged and sham-challenged mice. G, CTL assay for splenocytes isolated from vaccinated mice. H, growth of wild-type AB1 tumor in vaccinated mice (n = 5). Data represent mean ± SEM. ** P < 0.01. All experiments were replicated three times, with consistent data obtained.](image-url)
Figure 3. Therapeutic cure of mesothelioma-bearing mice after sPD1-p24fc/EP immunization. A, schematic representation for therapeutic study as color-coded for each group. B, two weeks after vaccination, p24-specific cellular (left) and humoral responses (right) in the peripheral blood of tumor-bearing mice (n = 5). C and D, tumor growth measurement (C) and survival curve (D) after therapeutic vaccination. E, representative BLIs of therapeutic treatment. F, dose-dependent effect of sPD1-p24fc vaccination in tumor regression (n = 5). G, AB1-GAG tumor-bearing mice (n = 5) were treated with combinations of 100 μg sPD1-p24fc/EP, 100 μg p24fc/EP, 200 μg anti-PD-1 mAb (i.p.), and 100 μg sPD1-p24fc/EP and assessed for tumor growth. Data represent mean ± SEM. *P < 0.05; **P < 0.01. Three independent experiments were performed for B-E.

As antigen-specific immune responses had dose-dependent characteristics (13), three groups of mice were immunized with sPD1-p24fc/EP at 100, 20, and 4 μg of DNA one day after AB1-GAG inoculation according to the same experimental schedule (Fig. 3A), respectively. As expected, therapeutic cure was reproduced in the 100 μg group (Fig. 3F and Supplementary Fig. S3A). Similar to p24fc/EP, 4 μg sPD1-p24fc/EP failed to reject established tumor, whereas the 20 μg group displayed partial efficacy at day 27 after the third vaccination. The EC50 value for sPD1-p24fc/EP efficacy against mesothelioma is calculated to be 23.95 μg DNA in mice.

Furthermore, previous studies showed that anti–PD-1 mAb or soluble PD-1 can suppress tumor growth by blocking PD-1 pathway (24). To understand such effects in our vaccine setting, we administered tumor-bearing BALB/c mice with anti–PD-1 mAb or sPD1-p24fc/EP alone or with p24fc/EP in combination with anti–PD-1 mAb or sPD1-p24fc/EP in parallel to sPD1-p24fc/EP (Fig. 3G). Anti–PD-1 mAb after the first injection resulted in partial tumor regression (1/5 mice in anti–PD-1 mAb-p24fc/EP group and 2/5 mice in anti–PD-1 mAb alone group, respectively; ref. 25). However, this effect was abolished when tumor developed in large volume during second and third vaccination. Administration of sPD1-p24fc/EP elicited limited immune responses with no benefits in terms of rejecting established tumors (13). Again, only sPD1-p24fc/EP eliminated established AB1-GAG malignant mesothelioma (Fig. 3G). These results suggest the need of enhancing antitumor immunity by vaccination besides blocking the immune checkpoint.

Vaccine-elicited CD8+ T cells are responsible for mesothelioma elimination

We next investigated the types of immune response that correlate with mesothelioma elimination. In the therapeutic setting (26), CD4+ or CD8+ T cells were depleted by i.p. injection of commercial anti-CD4 (YTS191.1) or anti-CD8 antibody (YTS169.4), respectively, starting 1 day before tumor inoculation and repeated weekly (Fig. 4A; refs. 15, 16). Depletion of CD4+ T cells before and during vaccination still resulted in tumor rejection, leading to complete elimination of tumor in a time-frame similar to isotype control (Fig. 4B). The efficacy of sPD1-p24fc/EP is, therefore, less dependent on CD4+ T cells. However,
the therapeutic effect of sPD1-p24fc/EP vaccination was abolished when CD8\(^+\) T cells were depleted, and all mice died in less than 30 days (Fig. 4B). Similar results were observed for CD4\(^+\) and CD8\(^+\) T cells in the prophylactic setting (Fig. 4C; ref. 17). After depletion of CD4\(^+\) T cells, sPD1-p24fc/EP–vaccinated mice were able to eliminate tumor growth effectively (Fig. 4D, \(P = 0.0096\)), achieving 100% survival (Fig. 4E). In contrast, only partial protection was observed after CD8\(^+\) T cells were depleted (Fig. 4D, \(P = 0.0384\)) with 40% survival (Fig. 4E).

To confirm these findings, we performed T-cell adoptive transfer experiments in immunodeficient SCID mice (18, 19). A total of 2 \times 10^6 CD4\(^+\) or CD8\(^+\) T cells purified from splenocytes of sPD1-p24fc/EP–vaccinated mice were injected intravenously 7 days after inoculation of AB1-GAG cells (Fig. 4F), respectively, when tumor size reached approximately 5 to 10 mm in diameter. As compared with p24fc/EP and PBS controls, mice that received sPD1-p24fc/EP–induced CD8\(^+\) T cells showed significant rejection of established solid tumors (Fig. 4G), likely owing to their potent cytotoxicity against AB1-GAG cells ex vivo starting from 0.2:1 E:T ratio (Fig. 4H) and led to 100% survival during the 44 days of the experiment (Fig. 4I, \(P < 0.01\)). To a much lower extent, adoptive transfer of CD4\(^+\) T cells resulted in slower tumor growth during the first 31 days and appeared to
relapse thereafter (Fig. 4). These cells showed modest cytotoxic effects starting at 2:1 E:T ratio (Fig. 4K) and led to 40% survival. The tumor protection we have observed so far was not due to antibody-dependent cell-mediated cytoxicity, as passive transfer of antisera derived from sPD1-p24fc/EP-vaccinated mice did not show any protective effects in SCID mice (Supplementary Fig. S3B). These findings were reproduced in another C57BL/6 melanoma model of GAG-transduced B16F10 cells (Supplementary Fig. S4A and B). B16F10-GAG tumor regression and enhanced survival was observed after adoptive transfer of CD8\(^+\) T cells isolated from 20 μg dose of sPD1-p24fc/EP but not p24fc/EP-vaccinated C57BL/6 mice (Supplementary Fig. S4C–S4E). Taken together, vaccine-elicited CD8\(^+\) T cells proved essential in eliminating mesothelioma and melanoma cells.

### Mechanism of vaccine-elicited CD8\(^+\) T cells in controlling mesothelioma

Previous studies have demonstrated that tumor cells could be eliminated by T-cell–dependent multifaceted killing effects (27). Consistently, sPD1-p24fc/EP–elicited CD8\(^+\) T cells did not produce stronger perforin and granzyme B upon antigen stimulation (13). However, CD8\(^+\) T cells of sPD1-p24fc/EP–vaccinated mice released significantly higher levels of IFNγ when cocultured with AB1-GAG cells in vitro, as compared with controls (Fig. 5A). Culture with these supernatants increased the fraction of AB1-GAG cells in G1 cell-cycle arrest by approximately 5% to 10% as compared with PBS group (Supplementary Fig. S5A). Similar effect was observed when culturing AB1-GAG cells with commercial IFNγ (Supplementary Fig. S5B), which was further enhanced when both IFNγ and TNFα were present (Supplementary Fig. S5C). Besides cytokine effects, direct killing through apoptosis pathways is involved in CD8\(^+\) T-cell–mediated cytoxicity. As AB1-GAG tumor cells express DR5 but not Fas on surface (Fig. 5B), we focused on the TRAIL-DR4/DR5 apoptotic pathway (28). Vaccination with sPD1-p24fc/EP resulted in selective upregulation of TRAIL on tetramer+CD8\(^+\) T cells (Fig. 5C). AB1-GAG cells were then cocultured with purified CD8\(^+\) T cells isolated from sPD1-p24fc/EP–vaccinated or control mice together with TRAIL blocking or control antibodies (Fas ligand blocking and isotype antibodies). Blocking of TRAIL reduced significantly the cytolytic activity of CD8\(^+\) T cells from sPD1-p24fc/EP–vaccinated mice (Fig. 5D).

To determine the functionality of T cells in vivo, we further assessed splenic T cells and TILs following vaccination of 100 μg sPD1-p24fc/EP or controls in tumor-bearing mice (Fig. 3A) and analyzed by flow cytometry (Supplementary Fig. S6). sPD1-p24fc/EP mice had significantly higher levels of anti–specific CD8\(^+\) T cells in their spleens at day 28 and 42 by tetramer staining (Supplementary Fig. S7A). However, in TILs, strikingly high (>80%) p24-tetramer+CD8\(^+\) T cells in both groups were observed, suggesting that a significant portion of T cells infiltrating tumors were specific for p24. Next, CD8\(^+\) T cells from both spleens and tumor were assayed for cytokine production at day 14, 28, and 42 after tumor challenge, which consistently showed reduced tumor size at day 42, the end point of observation before measurement of complete regression. sPD1-p24fc/EP led to significantly increased IFNγ and TNFα production by CD8\(^+\) T cells in both the spleen and TILs as compared with p24fc/EP control (Supplementary Fig. S7B). Furthermore, frequencies of polyfunctional CD8\(^+\) T cells that produce dual cytokines (TNFα and IFNγ) were substantially higher with sPD1-p24fc/EP in both spleen and TILs (Fig. 6A and Supplementary Fig. S7C). However, no difference was found for IL2 or IL10–expressing CD8\(^+\) T cells in either the spleen or tumor (Supplementary Fig. S8A). As differentiation and cytotoxicity of CD8\(^+\) T cells are regulated by transcription factors T-bet and eomesodermin (Eomes; ref. 29),
measured their expression besides GATA-3. Indeed, CD8\(^+\) T cells induced by sPD1-p24fc/EP expressed significantly higher levels of T-bet and Eomes in both spleen and TILs (Fig. 6A), but no difference in GATA-3 was observed (Supplementary Fig. S8A). Elevated IFN\(\gamma\)/TNF\(\alpha\), T-bet and Eomes in adoptively transferred sPD1-p24fc/EP-induced CD8\(^+\) T cells into tumor-bearing mice were also found (Fig. 6B), suggesting the importance of these cells for effective elimination of tumor cells (Fig. 4G).

**Vaccine-elicited CD8\(^+\) T cells overcome tumor-induced immunosuppression**

The establishment of an immunosuppressive environment is associated with tumor immune escape and development (12). We, therefore, sought to investigate this through measuring the expression of the surface molecules PD-1, Tim-3, and CTLA-4 on CD8\(^+\) T cells; PD-L1 and PD-L2 on tumor-associated cells; and proportions of Treg (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)) and MDSCs (CD11b\(^+\)Gr-1\(^+\)) among different treatment groups (Supplementary Fig. S6). As early as 7 days after inoculation, AB1-GAG resulted in significantly expanded PD-1\(^+\) and Tim-3\(^+\) CD8\(^+\) T cells in the spleens of nonvaccinated mice (Supplementary Fig. S9). Moreover, tumor development was associated with significantly increased frequencies of PD-1\(^+\) and Tim-3\(^+\) CD8\(^+\) T cells and of Treg and MDSC cells in p24fc/EP-vaccinated mice (Fig. 7A). In contrast, the frequencies of these immunosuppressive cells were significantly lower in sPD1-p24fc/EP-vaccinated mice (Fig. 7A). However, TACs showed higher PD-L2 expression in p24fc/EP-vaccinated mice compared to controls.

**Figure 6.** Enhanced effector function of vaccine-elicited CD8\(^+\) T cells. Assessment of CD8\(^+\) T cells after three vaccinations in BALB/c mice (n = 5; A) or adoptively transferred sPD1-p24fc\(-\)elicited CD8\(^+\) T cells into tumor-bearing SCID mice (n = 5; B) for the expression of IFN\(\gamma\)/TNF\(\alpha\), T-bet, or Eomes. Data represent mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Two independent experiments were performed.
Figure 7. Vaccine-elicited CD8⁺ T cells overcome tumor suppressive environment. Assessment of CD8⁺ T cells after three vaccinations in BALB/c mice (n = 5; A) or adoptively transferred sPD1-p24fc–elicited CD8⁺ T cells into tumor-bearing SCID mice (n = 5; B) for the expression of Tim-3 or PD-1, and frequencies of MDSC and CD4⁺ Tregs was also examined. C, expression of Tim-3 or PD-1 (left) and IFNγ or TNFα (right) production on tetramer⁺ tumor-infiltrating CD8⁺ T cells as compared with splenic CD8⁺ T cells at day 42 after AB1-GAG challenge from mice vaccinated with sPD1-p24fc. Two independent experiments were performed. Data represent mean ± SEM. **, P < 0.01; ***, P < 0.001. D, correlation analyses of antigen-specific CD8⁺ T cells or antigen-specific CD8⁺:MDSC ratio with tumor mass in adoptive transfer experiments.
compared with sPD1-p24a/EP (Fig. 7A and Supplementary Fig. S6C), in line with high PD-1+ CD8+ T cells, suggesting that PD-1/PD-L2–negative signaling could occur within the tumor microenvironment.

To investigate whether the surface expression of Tim-3 and PD-1 on CD8+ T cells reflects impaired functionality of cytokine production, we characterized the pattern of Tim-3 and PD-1 expression on both splenic and tumor-infiltrating tetramer-specific CD8+ T cells. Tetramer+ CD8+ T cells from tumor showed PD-1 and Tim-3 expression, whereas splenic tetramer+ CD8+ T cells were negative for PD-1 or Tim-3 (Fig. 7C, left and Supplementary Fig. S10A). Importantly, Tim-3+ or PD-1+ CD8+ T cells rendered lack of IFNγ and TNFα production, whereas PD-1+ Tim-3+ CD8+ T cells showed increased frequencies of IFNγ+TNFα+ population after repeated sPD1-p24a/EP vaccination (Supplementary Fig. S10B). In addition, tetramer+ Tim-3+ and tetramer+PD-1+ CD8+ T cells did not produce IFNγ or TNFα when infiltrated into tumor (Supplementary Fig. S10C), whereas splenic tetramer+ CD8+ T cells (Tim-3 or PD-1 negative) could still produce IFNγ and TNFα (Fig. 7C, right). Together, these data implied that PD-1 and Tim-3 expression on CD8+ T cells represents exhausted phenotypes in mice bearing AB1-GAG malignant mesothelioma.

To investigate whether the immunosuppressive cells could be associated with functional CD8+ T cells, correlation analyses were performed. Interestingly, in spleen, the frequency of tetramer+CD8+ T cells was inversely correlated with Treg (Supplementary Fig. S11A), whereas the frequency of MDSCs was positively correlated with either PD-1+CD8+ or Tim-3+CD8+ T cells (Supplementary Fig. S11B), suggesting that Treg and MDSC may contribute to the suppression of CD8+ T-cell function. As tumor mass is determined by the ratio of T-effectors to Tregs (30), we found that this ratio in either spleen or tumor was inversely correlated with tumor mass after sPD1-p24a/EP vaccination (Supplementary Fig. S11C). In same tissue compartments, we further found that the ratio of effector tetramer+CD8+/MDSCs was inversely correlated with tumor mass with stronger statistical significance (Supplementary Fig. S11D).

Next, to further illustrate the importance of sPD1-p24a/EP–elicited CD8+ T cells in overcoming the tumor immunosuppressive environment, adoptive transfer experiment confirmed that these cells alone retained low expression of Tim-3 in both spleen and tumor, low PD-1 in spleen but not in tumor, and elimination of MDSCs and PD-L2+ TACs (Fig. 7B), with the frequency of tetramer+CD8+ T cells inversely correlated with tumor mass (Fig. 7D, left). Inverse correlation was also found between the ratio of tetramer+CD8/ MDSCs and tumor mass in both spleen and tumor (Fig. 7D, middle and right). Consistently, the frequency of MDSCs was positively correlated with PD-1+CD8+ or Tim-3+CD8+ T cells in spleen (Supplementary Fig. S12A), which in turn pertain to weak TNFα production in spleen (Supplementary Fig. S12B) and TNFα and IFNγ production of TILs (Supplementary Fig. S12C). Collectively, sPD1-p24a/EP–elicited CD8+ T cells are essential in overcoming tumor-induced immunosuppression.

Discussion

In this study, we demonstrated that high frequency of vaccine-elicited effector CD8+ T cells plays a central role in preventing and curing malignant mesothelioma that expresses the cognate p24 antigen (Figs. 2–4). To our knowledge, this is the first study to show that active DNA vaccination alone elicits these protective CD8+ T cells with such efficacy by overcoming tumor-associated immunosuppression in an immunocompetent animal cancer model (Figs. 5–7). Moreover, vaccine-elicited CD8+ T cells with T-bet+, Eomes+, and IFNγ+TNFα+ phenotypes could retain their effector functions once infiltrated into tumor, reduce MDSC and Treg cell populations, and lead to the complete clearance of tumor cells. Importantly, only the sPD1-p24a/EP vaccine, but not conventional DNA vaccines, could elicit such CD8+ T cells with the properties of broad reactivity, long-term memory, polyfunctionality, and cytotoxicity (13). Thus, T-cell programming that could enhance the functionality of CD8+ T cells is an important aspect of tumor vaccine (31). Therefore, such an active vaccination using repeatable sPD1-based DNA/EP vaccine, a major advantage over live-vectored vaccines, can be further developed as a strategy for immunotherapy of cancer such as malignant mesothelioma. Our results warrant future investigation of cancer cure using combined active vaccination and immune checkpoint blockade (Fig. 3G).

sPD1-p24a/EP–elicited CD8+ T cells retain an active functional state after repeated vaccinations. Previous studies showed that tumor-infiltrating CD8+ T cells, as well as tumor antigen-specific T cells in nonsolid tumors, express a high level of PD-1 (11), which would be functionally impaired upon interaction with its ligands PD-L1/PD-L2 that are commonly expressed by the tumor and myeloid cells present in the tumor microenvironment (32). Here, we found that sPD1-p24a/EP–elicited CD8+ T cells expressed low PD-1 in the spleen, but acquired a high level of PD-1 expression similar to control vaccines once inside the tumor. Despite this, the frequencies of tumor-infiltrating polyfunctional IFNγ+ TNFα+ CD8+ T cells remained high (Fig. 5A and B). Moreover, PD-1 expression on TACs was significantly suppressed in sPD1-p24a/EP–vaccinated mice and by adoptively transferred CD8+ T cells (Fig. 7A and B:33,34), which suggests that PD-1/PD-L2 engagement has less chance to occur.

Recent studies indicated that exhausted CD8+ T cells also upregulate Tim-3 (35), particularly in melanoma (36), leukemia (37), colon carcinoma, prostate carcinoma, and sarcoma (38, 39). Here, we found that PD-1 and Tim-3 expression on tumor-infiltrating CD8+ T cells abolished their ability to produce both IFNγ and TNFα (Supplementary Fig. S10B). Furthermore, we consistently found strong inverse correlations between frequencies of Tim-3–CD8+ T cells and IFNγ+CD8+ or TNFα+CD8+ T cells, indicating that Tim-3 is a useful marker for the assessment of functional antitumor CD8+ T cells besides PD-1 (Supplementary Fig. S12B and S12C: ref. 39). Unlike PD-1, however, Tim-3 expression on CD8+ T cells after repeated sPD1-p24a/EP vaccinations remained low in tumor (Fig. 7A), suggesting that vaccine-elicited CD8+ T cells retained the active functional state without exhibiting this immunosuppressed phenotype, which was further indicated by high
levels of IFNγ+/TNFα+, T-bet, and Eomes expression (Fig. 6A and B; ref. 40). Further examination of p24 tetramer-specific CD8+ T cells in the spleen showed that TNFα and IFNγ are realized when PD-1 and Tim-3 expression remains low (Supplementary Fig. S10B and S10C). However, tumor-infiltrated tetramer+ CD8+ T cells upregulate PD-1 and Tim-3 and reduce TNFα and IFNγ production. As demonstrated in our adoptive transfer experiments (Fig. 4G–I), CD8+ T cells alone can reduce tumor volume, suggesting that these cells must be functional initially when they reach the tumor site. These results suggested that even most tetramer+ CD8+ T cells become functionally exhausted in the tumor (Fig. 7C; ref. 41); the continuous dynamic influx of functional CD8+ T cells resulting from repeated sPD1-p24g/EP vaccinations eventually overcome tumor microenvironment and eliminated the tumor. Besides, because sPD1-p24g/EP vaccination enhanced T-cell epitopic breadth (13), expanded CTL populations recognizing broader repertoire of antigens may also contribute to the protective effects (42).

This study also shows important characteristics for vaccine-elicted CD8+ T cells required to overcome the immunosuppressive tumor microenvironment established by high MDSC and Treg cells (12, 43). We found strong positive correlations between the frequencies of MDSC and PD-1+ CD8+ or Tim-3+ CD8+ T cells (Supplementary Figs. S11B and S12A), reaffirming the function of MDSCs in suppressing CD8+ T-cell function (32, 43). Interestingly, in sPD1-p24g/EP-vaccinated mice or mice receiving sPD1-p24g/EP+-elicited CD8+ T cells alone, MDSCs were significantly reduced in both spleen and tumor, which likely assisted the elimination of solid tumor through high levels of tetramer+ CD8+ T cells (Fig. 3 and Supplementary Fig. S7). This notion was supported by our results showing strong inverse relationships between tetramer+ CD8+ T cells or tetramer+ CD8+ T cells/MDSC ratio and tumor mass (Fig. 7D and Supplementary Fig. S11D). One enticing question is the mechanism by which sPD1-p24g/EP- induced CD8+ T cells eliminate MDSCs. On the one hand, the effective killing of tumor by these CD8+ T cells would reduce tumor-induced MDSCs (Figs. 4H and 7). On the other hand, polyfunctional CD8+ T cells infiltrating into the tumor could secrete multiple cytokines such as IFNγ and TNFα to perturb MDSC function or differentiation (Fig. 6; refs. 9, 13, 44). In addition, MDSCs can acquire soluble antigen and present to antigen-specific CD8+ T cells, which lead to T-cell tolerance by nitration of the TCR through MDSC-secreted peroxynitrite (45). sPD1-p24g/EP+-elicited CD8+ T cells may, in turn, kill the p24 peptide presenting MDSCs in a process similar to CTL killing of peptide-labeled DCs in vivo (46, 47). CD8+ T cell could also evade Treg suppression by direct killing (48), which was also reflected by our data that high level of antigen-specific CD8+ T cells correlated with low level of Treg cells (Supplementary Fig. S11A).

Finally, to test the vaccine in a more physiologic setting, we investigated tumor antigen WT1 (49) in the context of PD-1-based DNA vaccination (e.g., sPD1-WT1/EP). Our preliminary results showed neither the enhancement of WT1-specific CD8+ T-cell immune responses nor antitumor effect against WT1-expressing mesothelioma (data not shown), suggesting a limitation of such vaccine for immune recognition of this self-tumor antigen, likely due to antigen tolerance and T-cell clone depletion from self-antigen vaccination (50). Therefore, future investigations should evaluate modified self-tumor antigens or virus-encoded tumor antigens to generate highly reactive CD8+ T cells. In addition, given the great efficacy of sPD1-p24g/EP vaccine in tumor models, future studies should evaluate its application in eliminating HIV-infected cells including the latent reservoir.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

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