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Antimesothelioma Immunotherapy by CTLA-4 Blockade Depends on Active PD1-Based TWIST1 Vaccination

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Checkpoint immunotherapy is a major breakthrough for cancer treatment, yet its efficacy is often limited against many types of malignancies, including malignant mesothelioma. Considering that the immunotherapeutic efficacy depends on immunosurveillance, we sought to develop an active immunization method to break immune tolerance to tumor self-antigen. Here, we demonstrated that TWIST1, the basic helix-loop-helix transcription factor, was associated with human mesothelioma tumorigenesis and required for the invasion and metastasis of mesothelioma in the immune-competent murine AB1 model. When conventional TWIST1 vaccines were not effective *in vivo*, programmed cell death protein 1 (PD1)-based vaccination provided prophylactic control by inducing long-lasting TWIST1-specific T cell responses against both subcutaneous and metastatic mesothelioma lethal challenges. Furthermore, while CTLA-4 blockade alone did not show any immunotherapeutic efficacy against established mesothelioma, its combination with PD1-based vaccination resulted in 60% complete remission. Mechanistically, these functional T cells recognized a novel highly conserved immunodominant TWIST1 epitope, exhibited cytotoxic activity and long-term memory, and led to durable tumor regression and survival benefit against established AB1 mesothelioma and 4T1 breast cancer. We concluded that PD1-based vaccination controls mesothelioma by breaking immune tolerance to the tumor self-antigen TWIST1. Our results warrant clinical development of the PD1-based vaccination to enhance immunotherapy against a wide range of TWIST1-expressing tumors.

INTRODUCTION

Malignant mesothelioma is a lethal type of cancer linked to historical exposure to airborne asbestos that typically arises from the pleura. The incidence and mortality of mesothelioma continue to rise in developing countries primarily.[1] Treating malignant mesothelioma is challenging because the majority of patients (>75%) experienced relapse even after multimodality treatment (combined surgery, chemotherapy, and/or radiotherapy).[2] Chemotherapy with pemetrexed plus cisplatin has been the only approved regimen for more than a decade, but this approach only achieved modest benefits at best and many patients are unfit for such treatment.[3] Although antibodies targeting immune checkpoint molecules, such as cytotoxic T lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD1), and programmed death-ligand 1 (PD-L1), have improved therapeutic efficacy in certain cancers, their effects are unsatisfactory in patients with mesothelioma.[4] In particular, the first randomized phase III trial against mesothelioma using anti-CTLA-4 antibody failed to meet its primary end point of improved overall survival.[5,6] PD1 and PD-L1 checkpoint blockade antibodies have been shown some promising results in treating advanced mesothelioma in phase I and II trials, yet the overall responsive rate is below 30%.[7,8] In order to enhance the efficacy of existing immunotherapy, we speculate that it is still needed to elicit antitumor responses through active vaccination in mesothelioma patients.

Cancer vaccines involve boosting and proper activation of patients' own immune surveillance. It has been demonstrated that the number of tumor-infiltrating T cells directly correlates with improved clinical outcome in various cancer indications.[9,10] We and others have also reported that tumor-reactive cytotoxic T lymphocytes (CTLs) are essential for the cure of malignant mesothelioma.[11,12] Despite extensive efforts, however, therapeutic cancer vaccines still show few favorable outcomes in the establishment of clinical responses in advanced cancer patients, largely owning to the limited immunogenicity of tumor antigens within the immunosuppressive tumor microenvironment (TME).[13] In recent years, dendritic cell (DC)-based vaccine strategies have shown promising clinical efficacy. As a result, sipuleucel-T (Provenge), a DC-based cancer vaccine, has been approved as the first cancer vaccine by FDA for the treatment of metastatic prostate cancer.[14] Notably, using a DC-based vaccine as a maintenance therapy after the pemetrexed plus platinum chemotherapy induced an immunological CTL response for sustained tumor control in

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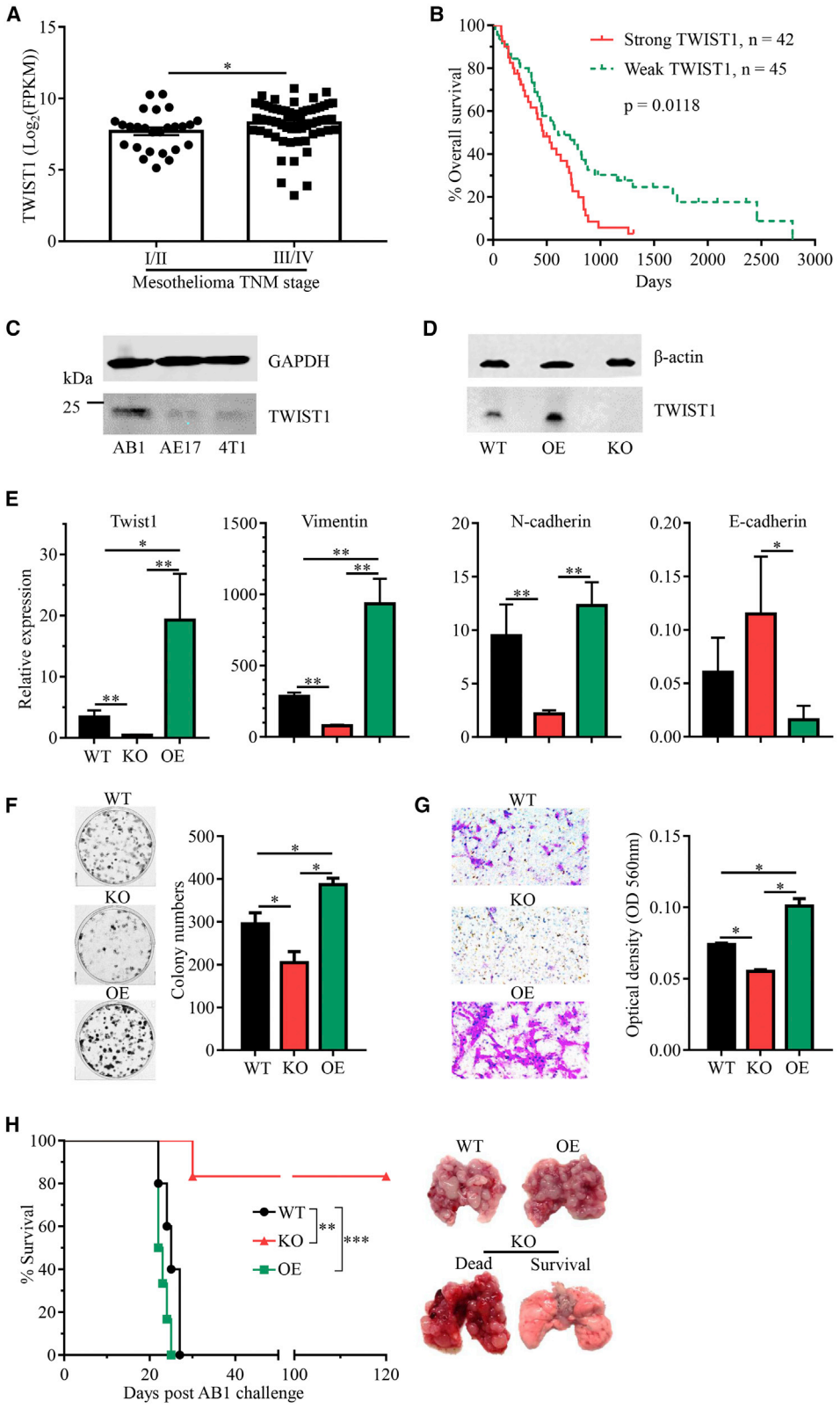

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mesothelioma patients, and this strategy is currently under evaluation in a phase III trial.[15,16] While these studies indicate the great therapeutic potential of DC-based vaccines, curing established malignancies is rare. Emerging studies suggested that combinational treatments using DC-based vaccination together with other cancer therapies to reverse immunosuppression may unleash fully the potential of DC-based cancer vaccines and improve patient survival.[1,3,13]

The frequent epithelial-mesenchymal transition (EMT) is an important feature of malignant mesothelioma.[17] Previous studies have demonstrated that high EMT level is closely related to increased mesothelioma metastasis and poor prognosis.[18,19] The basic helix-loop-helix transcription factor TWIST1 is one of the most critical factors that induces EMT and regulates the metastatic process of many solid tumors including melanoma, colon, breast, prostate, and gastric carcinomas.[20–23] Malignant mesothelioma was shown previously having upregulated TWIST1 expression.[24] However, the role of TWIST1 remains largely unknown in regulating mesothelioma EMT and pathogenesis.[25]

TWIST1, as a self-antigen, may serve as a tumor-associated antigen (TAA) for cancer vaccine. An previous study indicated that a recombinant yeast strain encoding full-length TWIST1 protein was capable of inducing both TWIST1-specific $CD8^+$ and $CD4^+$ T cell responses, reduced the size of primary transplanted 4T1 breast cancer, and had a greater antitumor effect on lung metastases.[26] Similarly, a recent study demonstrated that recombinant modified vaccinia Ankara (MVA) that expressed TWIST1 and co-stimulatory molecules OX40 inhibited breast cancer metastasis via a T-cell-dependent mechanism.[27] However, issues of preexisting immunity and safety may impede their implementation in cancer patients, and it also remains unknown whether or not TWIST1 vaccination could be an effective antitumor approach for mesothelioma treatment.

We previously reported that soluble PD1 (sPD1)-based DNA vaccination is a unique DC-targeting strategy that involves delivery of antigens to DC via PD1/PD-L interaction while triggering interleukin-12 (IL-12) production and antigen cross-presentation.[28] Using this vaccination strategy, we subsequently found that antigen-specific $CD8^+$ CTLs is capable of eliminating established mesothelioma expressing a xenoantigen.[11] Here, we hypothesize that effective sPD1-based TWIST1 DNA vaccine, namely sPD1-TWIST1, via *in vivo* electroporation (EP) would be able to break the immunotolerance to TWIST1 and elicit T cell responses directly against mesothelioma. We report that TWIST1 expression is associated with tumorigenesis in mesothelioma patients, and the protein is required for the invasion and metastasis of experimental AB1 mesothelioma in mice. Prophylactic sPD1-TWIST1 vaccination controls both subcutaneous and metastatic mesothelioma growth. Combined sPD1-TWIST1 vaccination and CTLA-4 immune checkpoint blockade further enhances TWIST1-specific T cell responses to provide therapeutic benefits in both mesothelioma and breast cancer models. The observed antitumor therapy is dependent on the vaccine-elicited TWIST1-specific long-term memory $CD8^+$ T cells that have great cytotoxicity potential and are uniquely elicited by the sPD1-TWIST1 vaccination against a highly conserved immunodominant short peptide. With the widespread expression of TWIST1 in different cancer types, sPD1-TWIST1 vaccination has high potential for cancer immunotherapy.

RESULTS

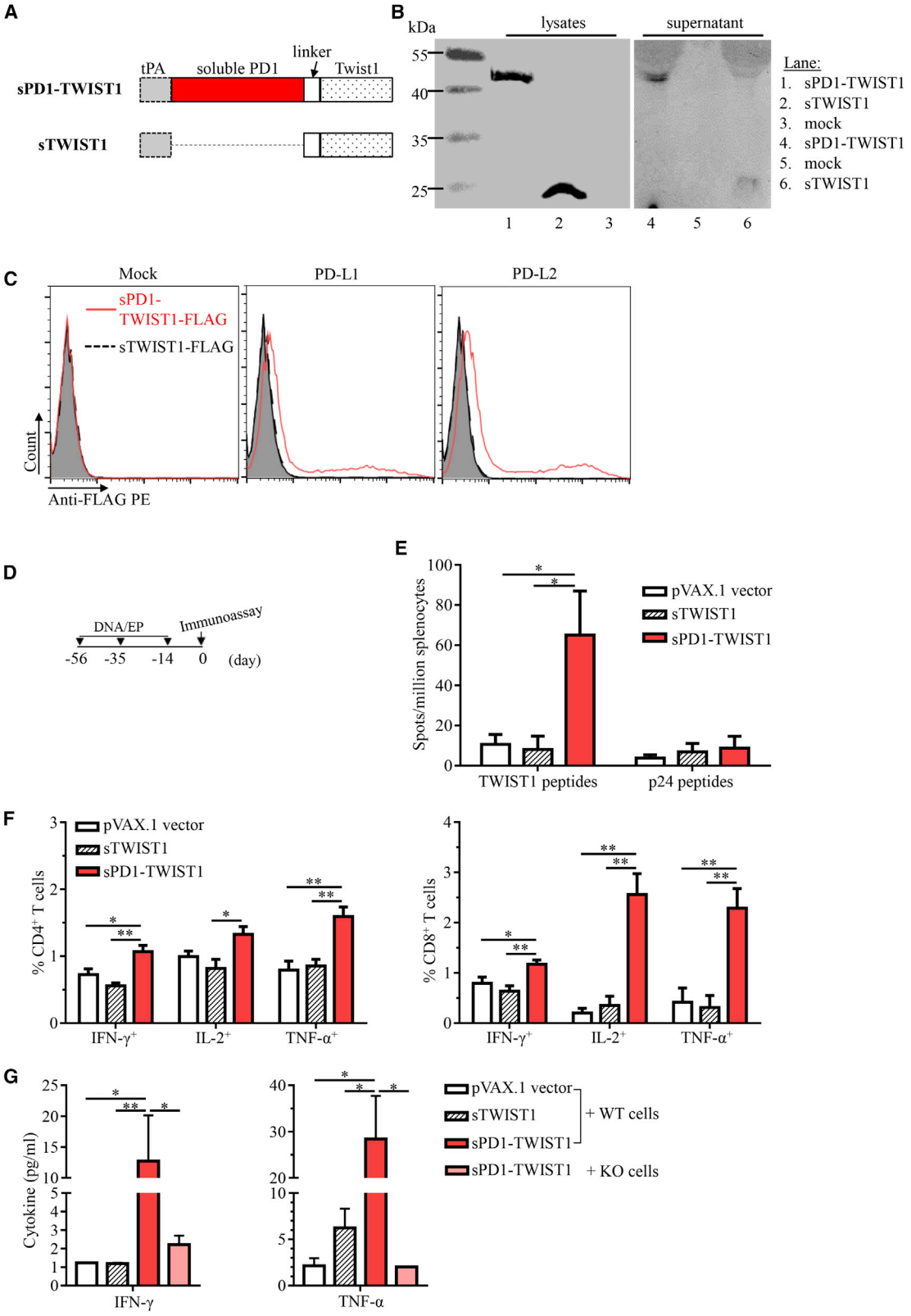
TWIST1 Expression Correlated with Mesothelioma Progression and Promoted Invasion and Metastasis of AB1 Mesothelioma

We initially investigated the effect of TWIST1 expression in human mesothelioma by comparing its expression level between different stages of 87 patients from the mesothelioma cohort (MESO) of The Cancer Genome Atlas (TCGA). Higher TWIST1 expression was found in patients with advanced-stage mesothelioma (TNM III and IV) as compared with early-stage tumors (TNM I and II) (Figure 1A). In addition, when the patients were stratified into two groups based on the TWIST1 expression in their tumors, patients with strong TWIST1 expression showed a significantly reduced overall survival (Figure 1B), suggesting an association of TWIST1 expression with mesothelioma tumorigenesis.

We next examined the expression of TWIST1 protein in two mesothelioma cell lines, AB1 and AE17, as well as in the 4T1 breast cancer cell line. Consistent with previous findings,[26] TWIST1 was detected in 4T1 cells (Figure 1C). Moreover, we found that both mesothelioma cell lines also expressed TWIST1 proteins with the highest expression level detected in AB1 cells. To explore the role of TWIST1 expression in AB1 mesothelioma development, we constructed AB1 cells in which TWIST1 expression was manipulated by either lentiviral vector-mediated overexpression or CRISPR/Cas9-mediated knockout (KO), respectively (Figure 1D). Using real-time qPCR, we found that overexpression of TWIST1 induced the expression of

Figure 1. Expression of TWIST1 Promotes Invasion and Metastasis of AB1 Mesothelioma

(A) TWIST1 expression in the mesothelioma cohort of TCGA (n = 87) by TNM stage. Stage I and II, n = 26. Stage III and IV, n = 61. (B) Kaplan-Meier overall survival curve of mesothelioma patients stratified by expression level of TWIST1, with weak (n = 45, TWIST1 $\leq$ 8.346) or strong (n = 42, TWIST1 > 8.346) expression of TWIST1. (C) Western blot analysis of TWIST1 in different murine tumor cell lines. The functional role of TWIST1 in AB1 cells was analyzed by gene overexpression (OE) and knockout (KO). (D) Western blot analysis of TWIST1 protein. WT, wild-type AB1 cells; OE, lentiviral vector-mediated TWIST1 OE; KO, CRISPR/Cas9-mediated TWIST1 KO. (E) qRT-PCR quantification of EMT-related molecules including vimentin, N-cadherin, and E-cadherin in WT, TWIST1 OE, or KO cells. Data shown are representative of two independent experiments. (F) Representative wells shown for colony-formation assay. (G) Matrigel cell invasion assay with representative images and quantification. Data in (F) and (G) shown are representative of two independent experiments. (H) Lung metastases after intravenous inoculation of 1×10^6 AB1 into BALB/c mice (n = 6). Left panel, survival curve. Right panel, representative images of lungs harvested at endpoint. Graphs show cumulative data from two separate experiments. Data represent mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.



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mesenchymal markers including vimentin, N-cadherin, fibroblast-specific protein 1 (FSP-1) and zinc finger E-box-binding homeobox 1 (ZEB1), as well as suppression of E-cadherin and occludin expression (Figure 1E; Figure S1A). This result suggested that TWIST1 may coordinate with other EMT transcriptional factors to promote EMT and metastasis of mesothelioma. Although TWIST1 overexpression or silencing did not alter the short-term proliferation of AB1 cells *in vitro* (Figure S1B), colony-formation efficiency of AB1 cells closely correlated with TWIST1 expression (Figure 1F). Specifically, overexpression cells showed enhanced clonogenic activity, while KO cells showed reduced activity. In line with their *in vitro* clonogenic activity, subcutaneous overexpression tumors exhibited comparably accelerated growth rate and significantly shortened survival time compared to subcutaneous KO tumors in syngeneic BALB/c mice (Figures S1C and S1D). We next sought to determine whether TWIST1 expression affects invasion and metastasis of AB1 mesothelioma. KO of TWIST1 expression profoundly decreased migration of AB1 cells, whereas its overexpression promoted it, in both Matrigel cell invasion assay (Figure 1G) and wound-healing migration assay (Figure S1E). To further verify the role of TWIST1 in driving mesothelioma metastasis, we established an *in vivo* metastasis model by intravenous (i.v.) injection of AB1 cells via the tail vein of BALB/c immunocompetent mice, which resulted in forming metastatic foci in the whole lungs, and humane euthanasia of all treated animals was required within 30 days due to clinical outcomes (Figure 1H). Using this model, we found that, compared to wild-type (WT) cells, TWIST1 overexpression enhanced metastatic activity of AB1 cells, while KO significantly suppressed their ability to metastasize to the lungs and prolonged tumor-free survival of animals. Overall, these findings support the notion that TWIST1 is an important transcription factor underlying mesothelioma invasion, metastasis, and tumor progression, suggesting that TWIST1 may serve as a therapeutic target to arrest cancer growth and metastasis.

PD1-Based Vaccination Enhanced TWIST1-Specific T Cell Responses

In order to determine whether sPD1-based fusion DNA vaccine would enhance TWIST1-specific antimesothelioma immunity, we first generated DNA vaccine construct encoding a fusion protein linking sPD1 with TWIST1 (sPD1-TWIST1) to compare with a conventional sTWIST1 DNA vaccine (Figure 2A). Expression of encoding TWIST1 proteins from these two constructs was confirmed by western blot analysis (Figure 2B). Importantly, while both TWIST1 proteins could be secreted as soluble forms, only sPD1-TWIST1 interacted with PD-L1/L2-expressing cells (Figures 2B and 2C), suggesting that sPD1-based TWIST1 vaccine may improve adaptive T cell immunity by targeting TWIST1 antigen to DCs as previously indicated.[11,28] To test this, we sought to determine whether sPD1-TWIST1 could enhance TWIST1-specific immune responses *in vivo* in BALB/c mice. In brief, 100 μg DNA plasmid of either sPD1-TWIST1 or sTWIST1 were injected intramuscularly (i.m.) via EP three times at 3-week intervals, as we have previously established.[11,28] Two weeks after the last vaccination, all mice were sacrificed, and blood and spleen specimens were collected for immune response analysis (Figure 2D). We found that sPD1-TWIST1 vaccination significantly elevated TWIST1-specific T cell responses compared to sTWIST1 vaccination (Figure 2E). Moreover, sPD1-TWIST1-vaccinated mice had substantially higher frequencies of interferon-γ (IFN-γ), IL-2-, and tumor necrosis factor alpha (TNF-α)-expressing $CD4^+$ and $CD8^+$ T cells after *ex vivo* stimulation with the TWIST1 peptide pool (Figure 2F; Figure S2), indicating that the sPD1-based vaccine breaks tolerance to the TWIST1 self-antigen. Importantly, when compared to controls, $CD3^+$ T cells of sPD1-TWIST1-vaccinated mice released significantly higher amounts of IFN-γ and TNF-α when co-cultured *in vitro* with WT AB1 cells, but not with TWIST1 KO cells (Figure 2G), thus demonstrating the specificity of vaccine-elicited T cells in recognizing TWIST1-expressing tumor cells. Taken together, our results support the notion that the sPD1-TWIST1 vaccine is useful to break TWIST1 immunotolerance for generating functional T cells specific for TWIST1-expressing mesothelioma.

PD1-Based Vaccination Inhibited Growth of Primary AB1 Mesothelioma

In order to evaluate the ability of TWIST1 vaccination to control tumor growth *in vivo*, a lethal dose of 1×10^6 WT AB1 cells were inoculated subcutaneously (s.c.) into vaccinated mice 2 weeks after the third vaccination (Figure 3A). Similar to our previous report showing the high efficacy of sPD1 DNA vaccination against mesothelioma,[11] we here found that the sPD1-TWIST1 vaccine significantly inhibited AB1 mesothelioma growth compared to the sTWIST1 or mock vaccine (Figure 3B). Furthermore, only the sPD1-TWIST1-vaccinated mice substantially prolonged the survival time of AB1-challenged mice, leading to 37.5% tumor-free survival (Figure 3C). To further demonstrate that the antitumor response is indeed TWIST1 specific, sPD1-TWIST1-vaccinated mice were challenged with TWIST1 overexpression, KO, or WT AB1 cells, respectively. Again, rejection of inoculated WT AB1 cells were observed in 2/5 animals, with 40% tumor-free survival (Figures 3D and 3E). In addition, sPD1-TWIST1

Figure 2. PD1-Based Vaccination Enhanced TWIST1-Specific T Cell Responses

(A) Schematic representation of TWIST1 DNA vaccine constructs. tPA, tissue plasminogen activator signal sequence. (B) Expression of TWIST1 DNA vaccine constructs after transfection in 293T cells. Cell lysates or culture supernatant of transfected 293T cells were subjected to western blot analysis using anti-TWIST1 antibody. (C) Flow cytometric analysis of binding between soluble proteins and mouse PD-L1/L2-transfected 293T cells. Transfection supernatant collected from sPD1-TWIST1-FLAG (red solid line)-, sTWIST1-FLAG (black dashed line)-, or mock (shaded region)-treated 293T cells were used to incubate 293T cells transiently transfected with mouse PD-L1 or PD-L2 expression vectors. Data shown are representative of two independent experiments. (D) DNA vaccination schedule. Groups of BALB/c mice (n = 4) received three DNA/EP vaccinations before being sacrificed for immunoassay at 2 weeks after the last vaccination. (E) ELISpot analysis of TWIST1-specific T cell responses. (F) Intracellular staining of IFN-γ-, IL-2-, and TNF-α-producing $CD4^+$ and $CD8^+$ T cells after DNA/EP vaccination. (G) Cytokine production following incubation of purified $CD3^+$ T cells with WT or TWIST1 KO AB1 cells. Data in (E)–(G) shown are representative of two independent experiments. Data represents mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

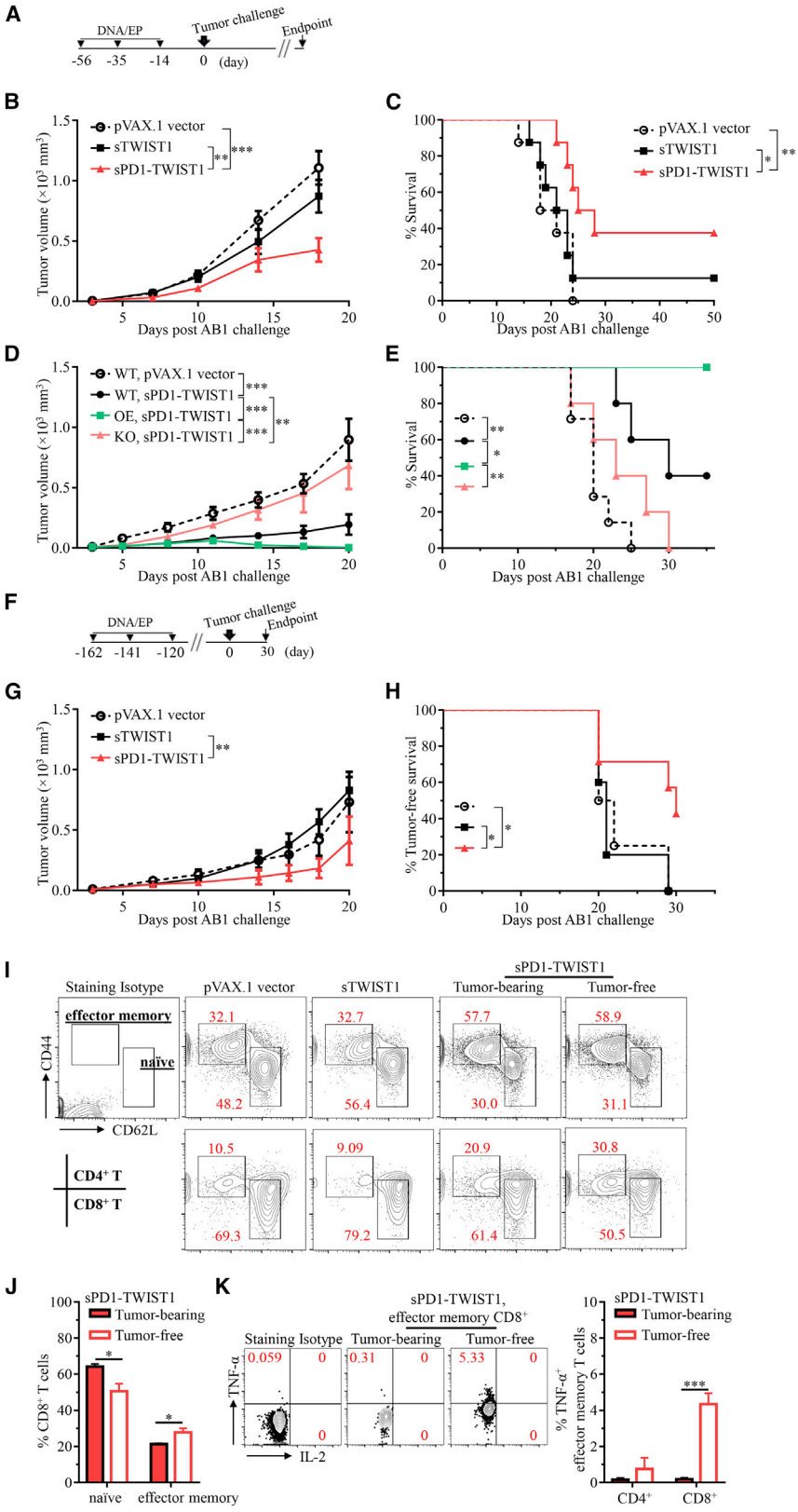


Figure 3. PD1-Based Vaccination Inhibited Growth of Primary AB1 Mesothelioma

(A) Schematic representation of treatment schedule. (B and C) Vaccinated mice (n = 8) were assessed by tumor growth (B) and survival (C) after WT AB1 challenge. Graphs show cumulative data from two separate experiments. Alternatively, sPD1-TWIST1-vaccinated mice (n = 5 each group) were challenged s.c. with 1×10^6 TWIST1 OE, KO, or WT AB1 cells, respectively. (D and E) Tumor growth (D) and survival (E) were calculated. (F) 120 days after the last vaccination, mice were challenged with 1×10^6 WT AB1 cells and measured for tumor growth (G) and tumor-free survival (H). (I) Mice were sacrificed at day 30 post-tumor-inoculation for analysis of naive ($CD44^{lo}CD62L^{hi}$) and effector memory ($CD44^{hi}CD62L^{lo}$) splenic T cells. (J and K) Tumor-free (n = 3) and tumor-bearing (n = 4) mice receiving sPD1-TWIST1 vaccination were assessed by frequencies of $CD8^+$ naive and effector memory T cells (J) and production of TNF-α and IL-2 in $CD4^+$ and $CD8^+$ effector memory T cells (K). Numbers within each plot represent cell proportions. Data represents mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

vaccination was proved to be significantly more efficient at eliminating implanted TWIST1 overexpression tumors than WT tumors, which resulted in complete rejection 17 days post-challenge. In contrast, loss of TWIST1 expression completely diminished the antitumor activity of sPD1-TWIST1 vaccination, whereas all mice developed tumors and died within 30 days. Therefore, these results ruled out any possible contribution of sPD1-mediated host immune activation to antitumor efficacy and demonstrated the pivotal role of sPD1-based vaccine in generating TWIST1-specific protective immunity against mesothelioma. Critically, since the persistence of induced long-lasting memory T cell immunity is the major goal of active immunotherapy against cancer, immunized mice were challenged with WT AB1 cells 120 days after the last vaccination to assess antitumor memory T cell responses (Figure 3F). We found that only sPD1-TWIST1-vaccinated mice inhibited WT tumor growth to achieve 3/7 tumor-free survival 30 days post-challenge, when all the mice in control groups (sTWIST1 and sham-vaccination) developed tumors (Figures 3G and 3H). At this time point, the abundance of splenic naive ($CD44^{lo}CD62L^{hi}$) and effector memory ($CD44^{hi}CD62L^{lo}$) T cell subsets were compared (Figure S3A). We found that sPD1-TWIST1-vaccinated mice constantly showed increased frequencies of effector memory T cell subsets for both $CD4^+$ and $CD8^+$ T cells (Figure 3I; Figure S3B). In particular, while effector memory $CD4^+$ T cells were maintained at similar levels between tumor-bearing and tumor-free mice in sPD1-TWIST1-vaccinated mice (Figure S3C), tumor-free mice had increased frequencies of effector memory $CD8^+$ T cells (Figures 3I–3J), which were capable for producing TNF-α (Figure 3K), suggesting a potential role of these cells in antitumor efficacy. Collectively, these results demonstrated that sPD1-TWIST1 vaccine elicited long-lasting adaptive immunity to provide protection against mesothelioma.

sPD1-TWIST1 Vaccine Inhibited AB1 Lung Metastasis

Since the expression of TWIST1 was responsible for mesothelioma metastatic activity, we next sought to study the activity of TWIST1 vaccines in inhibiting metastasis of AB1 mesothelioma cells. Using the same metastasis model described above, vaccine-immunized mice were injected i.v. with WT AB1 cells stably transduced with firefly luciferase (AB1-Luc) to induce pulmonary metastasis and pulmonary tumor growth was monitored by bioluminescence imaging. Compared to mock vaccination, the non-targeting sTWIST1 DNA vaccine failed to show any antimesothelioma activity (Figures 4A and 4B). Significant reduction of lung metastasis was only found in sPD1-TWIST1-vaccinated mice. At 28 days post-AB1-injection, mice in sTWIST1-vaccinated and mock-treated groups showed significantly reduced body weight (Figure 4C). Importantly, in line with *in vivo* imaging results, sPD1-TWIST1-vaccinated mice had significantly reduced metastasized nodules on the lung surface (Figure S4A) and fewer metastatic areas in the lungs (Figure 4D), suggesting a strong efficacy of sPD1-TWIST1 in inhibiting metastasis of AB1 mesothelioma. It has been reported that the establishment of an immunosuppressive environment is associated with tumor immune escape and mesothelioma development.[11,29,30] To illustrate the importance of vaccine-elicited T cell immunity in overcoming the mesothelioma-associated immunosuppressive environment in this metastasis model, we analyzed the T cell functionality as well as frequencies of various immunosuppressive cells *ex vivo* at the experimental end point (Figure S4B). We found that sPD1-TWIST1-vaccinated mice had significantly higher levels of IFN-γ- and TNF-α-producing $CD4^+$ and $CD8^+$ T cells (Figure 4E), as well as nautral killer (NK) cells (Figure S4C) compared to controls. Furthermore, frequencies of immunosuppressive cells, including the polymorphonuclear (PMN)- and monocytic (M)-myeloid-derived suppressor cells (MDSCs) subsets and $CD4^+$ regulatory T cells (Treg), were significantly lower in sPD1-TWIST1-vaccinated mice than the sTWIST-1- or mock-vaccinated mice (Figure 4E). Collectively, these results reinforced the notion that sPD1-TWIST1 vaccine construct was essential to break TWIST1 tolerance and demonstrated that sPD1-TWIST1 vaccination inhibited metastasis of AB1 mesothelioma and reduced tumor-associated immunosuppression.

Checkpoint Modulation Enhances the Antitumor Activity of sPD1-TWIST1 Vaccination for Curing Established Mesothelioma

Given the success of antibody-mediated immune checkpoint blockade in relieving immunosuppression of endogenous antitumor T cell responses in tumor-burdened hosts, we asked whether antimesothelioma responses generated by the sPD1-TWIST1 vaccine can benefit from checkpoint blockade. Since AB1 mesothelioma tumorigenesis upregulated the expression of both CTLA-4 and PD1 on T cells (Figure S5), we hypothesized that anti-CTLA-4 antibody (α-CTLA-4) may enhance the antitumor activity of sPD1-TWIST1 vaccination.[31] To test this hypothesis, we initially investigated whether α-CTLA-4 treatment could enhance activation of TWIST1-specific T cells in a bone-marrow derived DC (BMDC)-T cell co-culture system. We observed increased proliferation of T cells in co-cultures when BMDCs were pulsed with TWIST1 epitopes (Figure 5A), indicating the activation of TWIST1-specific T cells. Meanwhile, α-CTLA-4 significantly altered BMDC induced T cell activation in a dose-dependent manner in co-culture with $CD3^+$ T cells of sPD1-TWIST1-vaccinated tumor-bearing mice, but not of sTWIST1- or sham-vaccinated tumor-bearing mice (Figures 5A and 5B). Next, we studied the antitumor efficacy against established AB1 mesothelioma *in vivo*. Mice were first inoculated s.c. with the lethal dose of 5×10^5 AB1 cells, followed by sTWIST1, sPD1-TWIST1, or sham vaccination three times in 10-day intervals, starting at 7 days post-tumor-inoculation when the solid tumors were palpable (Figure 5C). At the same time, α-CTLA-4 at a dose of 200 μg per injection was administrated intraperitoneally (i.p.) starting from 8 days post-tumor-inoculation and every 4 days afterward. We found that the α-CTLA-4 monotherapy did not show any antitumor activity against established AB1 mesothelioma and all the mice needed to be euthanized within 40 days due to their clinical outcomes (Figures 5D and 5E). sPD1-TWIST1 vaccination monotherapy displayed modest antimesothelioma activity with slow tumor growth and resulted in tumor regression in 1/6 mice. Notably, combined therapy of sPD1-TWIST1 vaccination and α-CTLA-4 caused retarded tumor growth and there was a significant

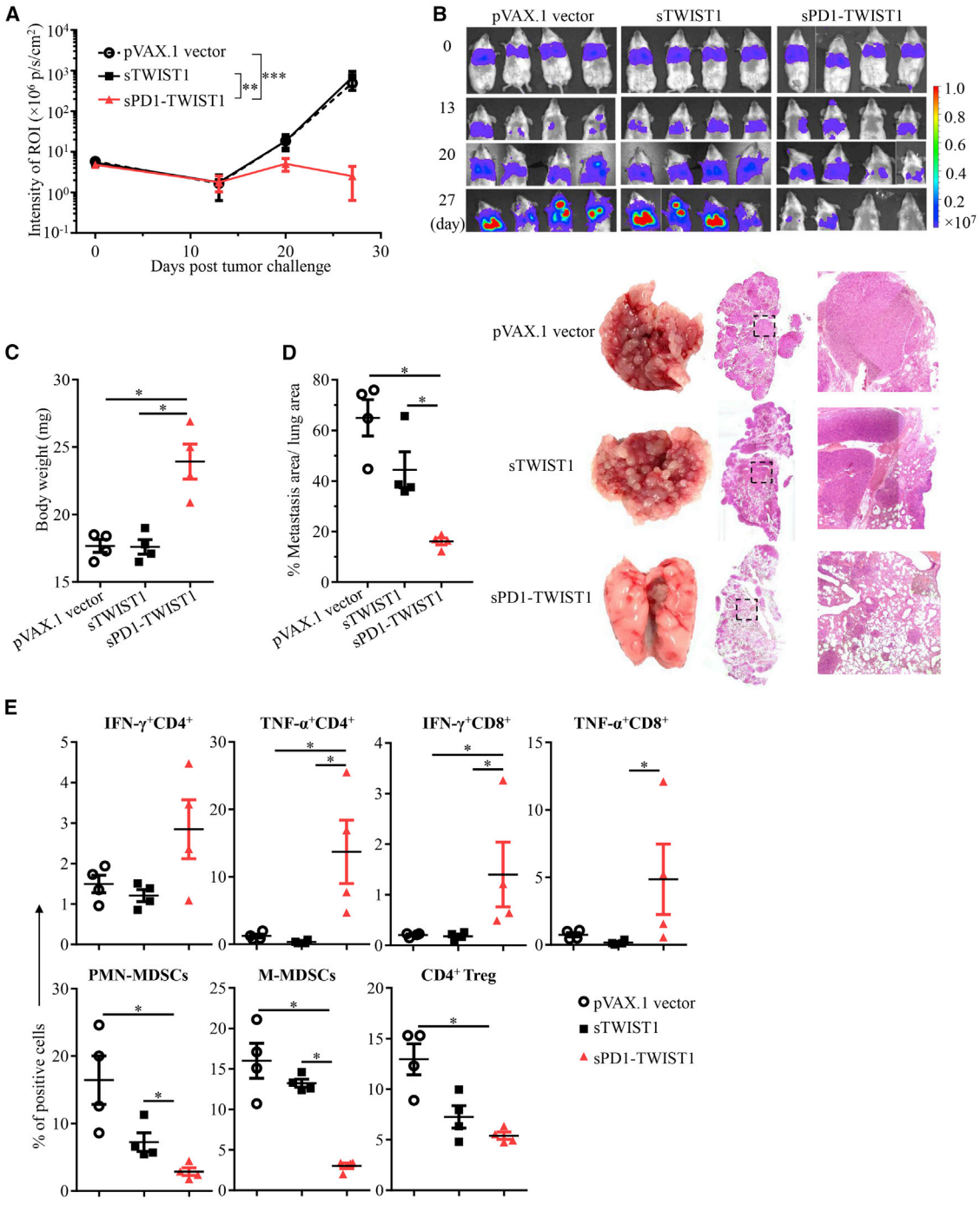
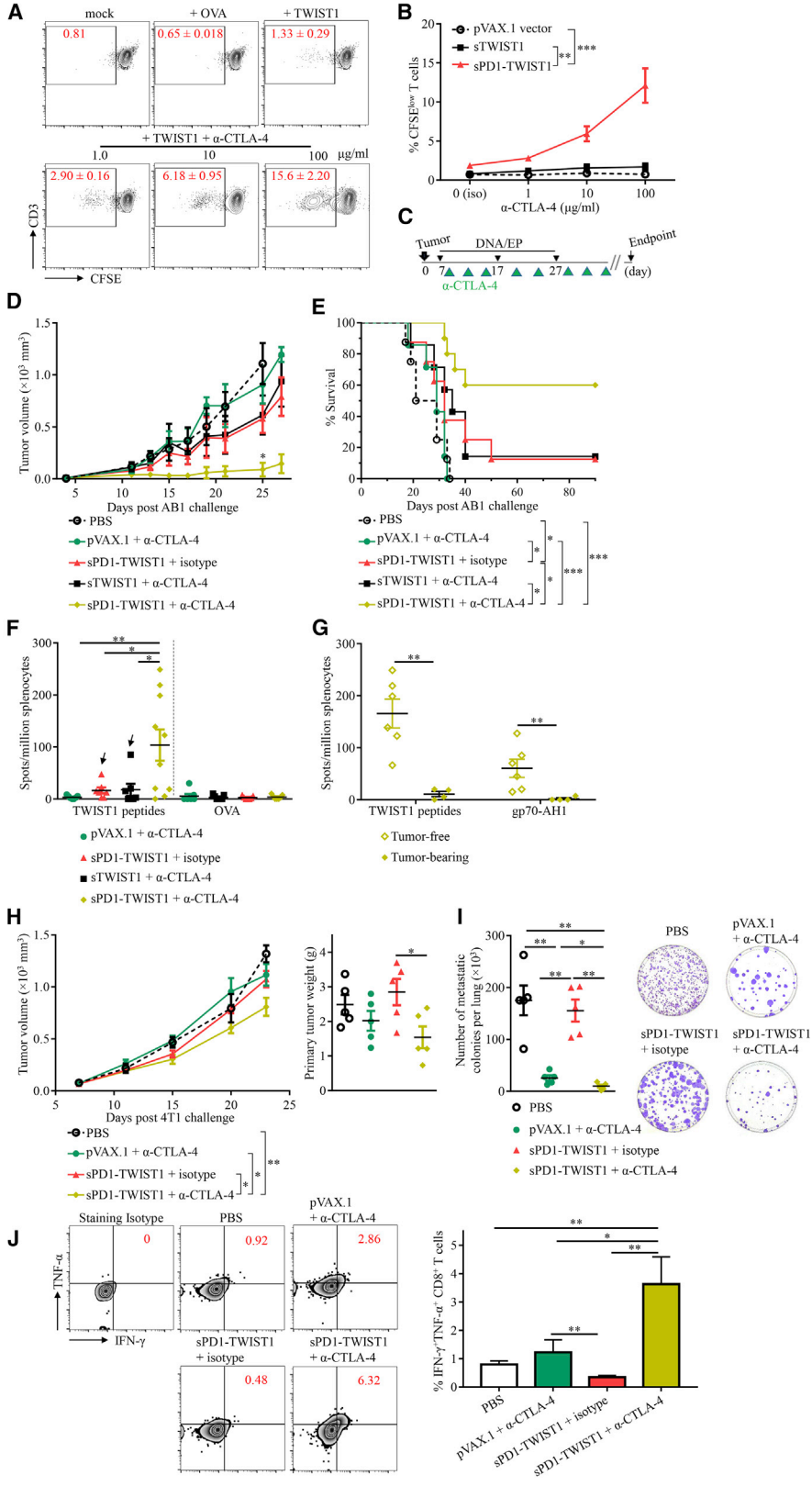


Figure 4. sPD1-TWIST1 Vaccine Inhibited AB1 Lung Metastasis

(A and B) Following the same vaccination regimen in Figure 3A, vaccinated BALB/c mice (n = 4) received 1 × 10^6 AB1 cells i.v. 2 weeks after last vaccination, and tumor growth (A) was monitored by bioluminescence and shown with representative bioluminescence images (B). (C and D) On day 28, the body weight of mice was measured (C), and then mice were sacrificed to collected lungs for (D) macroscopic evaluation and H&E staining. (E) Assessment of effector function of T cell subsets (upper) and immunosuppressive cell subsets (lower) in spleen at the endpoint. Data represents mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.



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reduction in tumor volume compared to both α-CTLA-4 monotherapy and PBS-treated groups, as measured at 25 days post-tumor-inoculation ($p = 0.0224$, compared to α-CTLA-4 monotherapy; $p = 0.0386$, compared to PBS). The combined therapy of PD1-based vaccine with α-CTLA-4 treatment also led to tumor eradication in 6/10 of the mice while sTWIST1 and α-CTLA-4 combined therapy failed to show significant enhancement on animal survival with only 1/7 tumor-free survival at the endpoint (Figures 5D and 5E). This result demonstrated the critical role of sPD1-TWIST1 vaccination in eliciting efficacious antitumor responses. We then determined the TWIST1-specific T cell response in these mice and found that splenocytes from the sPD1-TWIST1 vaccination and α-CTLA-4 combined therapy elicited more TWIST1-specific T cells, whereas neither sTWIST1 vaccination and α-CTLA-4 combined therapy nor monotherapies can do so (Figure 5F). Notably, mice with tumor eradication elicited more potent TWIST1-specific IFN-γ T cell responses than tumor-bearing mice (Figure 5G), demonstrating the involvement of vaccine-elicited T cell responses in clearing established AB1 mesothelioma. In addition to TWIST1, tumor-free mice also developed vigorous T cell responses against an immunodominant CTL epitope expressed in AB1 mesothelioma cells, gp70-AH1,[32] suggesting the induction of secondary tumor-specific cell responses following tumor eradication in these animals. In a separate model, previous studies have demonstrated principally the role of TWIST1 in 4T1 breast cancer metastasis and TWIST1 vaccination for breast cancer immunotherapy.[26,27] We found that the combined therapy of sPD1-TWIST1 and α-CTLA-4 reduced tumor growth and size of primary 4T1 mammary tumors and had a more potent antitumor effect against 4T1 lung metastases (Figures 5H and 5I), which was accompanied by markedly increased frequency of IFN-γ^+ TNF-α^+ $CD8^+$ T cells *in vivo* (Figure 5J). Taken together, these results demonstrated that administration of checkpoint modulator α-CTLA-4 significantly enhanced antitumor activity elicited by sPD1-TWIST1 vaccination, inducing regression of established AB1 mesothelioma as well as growth inhibition of mammary and metastatic 4T1 breast cancer.

Combination Therapy Induced Durable T Cell Immunity Responsive to an Immunodominant TWIST1 Peptide

We next sought to study the durability of the antitumor response induced by combined sPD1-TWIST1 and α-CTLA-4. In another group of mice that had eliminated AB1 mesothelioma after receiving the combined therapy of sPD1-TWIST1 and α-CTLA-4, an additional higher dose of 1×10^6 AB1-Luc cells were rechallenged s.c. on their contralateral flank >90 days after the initial complete tumor rejection. Complete rejection of AB1-Luc mesothelioma was observed 21 days later in these mice, while all naive mice succumbed to AB1-Luc challenge (Figures 6A and 6B), suggesting the induction of prolonged memory responses. In order to dissect the types of T cells responsible for mesothelioma elimination, an *in vitro* cytotoxicity assay was performed with purified splenic T cells from sPD1-TWIST1-vaccinated mice after initial complete tumor rejection. Consistent with IFN-γ ELISpot responses described above, $CD3^+$ T cells from mice receiving combined sPD1-TWIST1 and α-CTLA-4 showed enhanced *in vitro* cytotoxic activity in comparison to either the sTWIST1 combined therapy (Figure 6C) or monotherapies (Figure S6A). More importantly, the killing of target cells was performed by $CD8^+$ T cells, but not $CD4^+$ T cells (Figure 6C). In addition, adoptive transfer of these cytotoxic $CD8^+$ T cells resulted in retarded tumor growth and prolonged survival in SCID mice bearing AB1-Luc tumors (Figure 6D), demonstrating the critical role of the combined therapy in inducing efficacious antimesothelioma CTLs. We next characterized the TWIST1 amino acid sequences recognized by T cells induced by combined sPD1-TWIST1 and α-CTLA-4. *Ex vivo* isolated splenocytes were first screened in an ELISpot assay with minipools containing three 15-mers spanning the entire TWIST1 protein. And specific reactivity was mostly found against the minipool 37–39 (DKLSKIQTLKLAAR YIDFLYQVL) (Figure 6E; Figure S6B). In contrast, no response was found against minipool 40–42 (ARYIDFLYQVLQSDELDSKMASC), which contains the previously reported epitope LYQVLQSDEL.[26,33] We then tested the individual single 15-mers in the two minipools and found that peptides 37 and 38 showed stronger activity than peptide 39, suggesting that peptides 37–38 (DKLSKIQTLKLAAR YIDFL) contain the immunodominant epitope in BALB/c mice (Figure 6F; Figure S6B). Notably, this sequence is highly conserved across different host species. Therefore, these data demonstrated that combined therapy elicited vaccine-specific and durable antitumor $CD8^+$ CTLs responsive to an immunodominant short epitope within TWIST1 protein.

Figure 5. Checkpoint Modulation Enhances the Antitumor Activity of sPD1-TWIST1 Vaccination for Curing Established Mesothelioma

BMDCs were pulsed with TWIST1 or OVA peptides, followed by incubation with 1.0, 10, or 100 μg/mL α-CTLA-4 antibody. $CD3^+$ T cells purified from splenocytes of sPD1-TWIST1-, sTWIST1-, or sham-vaccinated tumor-bearing mice (Figure 3B) were added into the co-culture. Proliferation of T cells were analyzed from 6-day BMDC-T cell co-cultures. (A) Representative plots showing proliferation of $CD3^+$ T cells from sPD1-TWIST1-vaccinated mice. Numbers in each plot indicating $CFSE^{low}$ populations. (B) Proliferation of $CD3^+$ T cells from sPD1-TWIST1, sTWIST1, or sham-vaccinated mice. iso, mouse IgG_{2b} isotype control. Graphs show cumulative data from two separate experiments. (C) Schematic representation of therapeutic study. (D and E) Tumor growth measurement (D) and tumor-free survival curve (E) after therapeutic vaccination. Mice were sacrificed when tumor size was >15 mm. (F and G) TWIST1-specific T cell responses across all groups (F) or TWIST1-, AH1-specific T cell responses in combined sPD1-TWIST1 vaccination and α-CTLA-4 therapy group (G) were quantified by IFN-γ ELISpot assay. Arrow indicated individual tumor-free mouse in that group. Graphs show cumulative data from two separate experiments. At least eight mice were used in each group. Groups of female BALB/c mice ($n = 5$) were inoculated with 2×10^5 4T1 cells in the mammary gland, followed by vaccination three times in 10-day intervals, starting at 1 day post-tumor-inoculation. Anti-CTLA4 antibody at a dose of 200 μg per injection were administrated i.p. starting from 2 days post-tumor-inoculation and every 4 days afterward. (H) 4T1 Primary tumor growth curve (left) and tumor weights (right) harvested at day 27 post-4T1-inoculation. (I) Enumeration of clonogenic metastatic cells in the lungs (left) and representative images of clonogenic colonies after 14 days incubation (right, ×200 dilution factor). (J) Representative dot plots and percentages of IFN-γ^+TNF-α^+ $CD8^+$ T cells in spleens were measured at the endpoint. Data represents mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

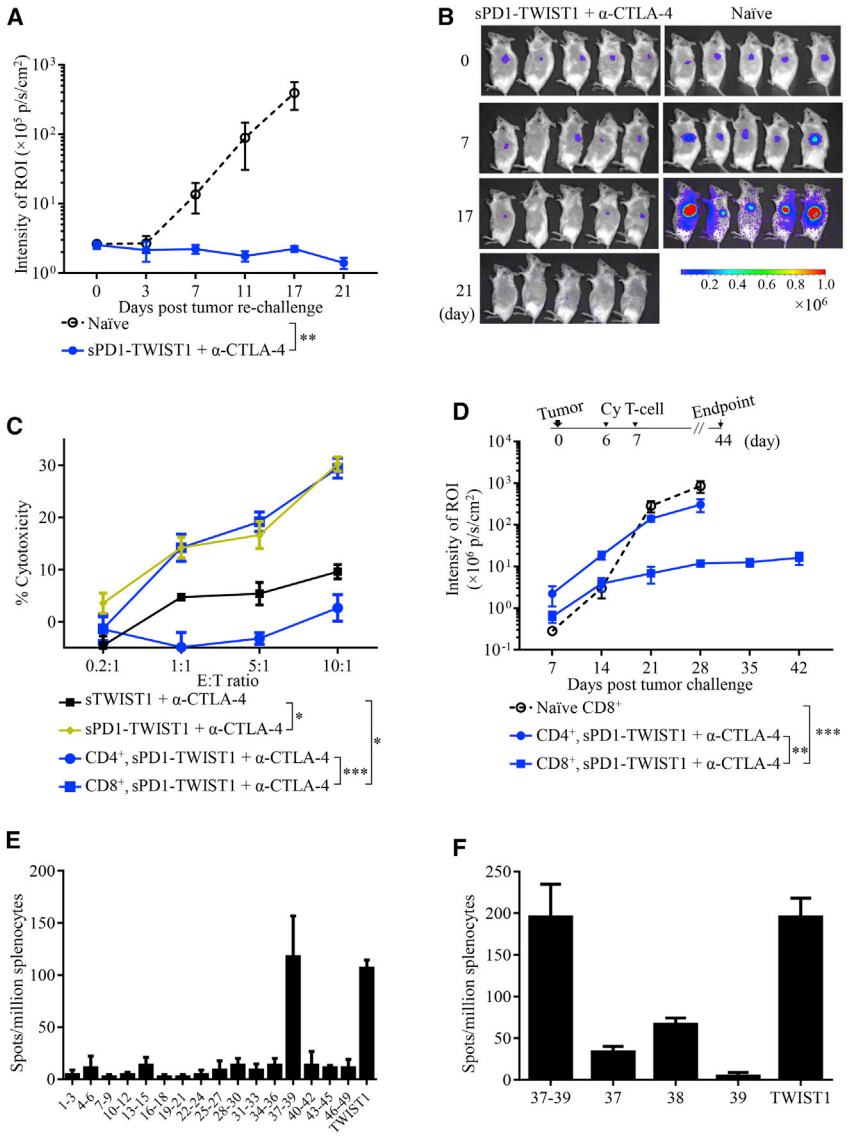

Figure 6. Combination Therapy Induced Durable T Cell Immunity Responsive to an Immunodominant TWIST1 Epitope

(A and B) 60 days after tumor ablation, protected mice (n = 5) in the combined sPD1-TWIST1 and α-CTLA-4 group were rechallenged s.c. and measured for tumor growth (A) with representative bioluminescence images of AB1-Luc tumors (B). (C) Cytotoxicity assay of T cells toward AB1 cells at different effector:target (E:T) ratios. T cells were isolated from spleen of mice receiving combined sPD1-TWIST1 vaccination and α-CTLA-4 therapy after initial complete tumor rejection. The experiment was repeated two times. (D) Schematic representation (upper) and tumor growth curve (lower) for T cell adoptive transfer. T cells from either naive or vaccinated/protected mice were adoptively transferred to SCID mice bearing 7-day-old AB1-Luc tumors and assessed for tumor growth. Cy (cyclophosphamide) at a dose of 150 mg/kg was administrated i.p. at day 6. (E and F) Characterization of TWIST1 immunodominant epitopes using minipools spanning the entire TWIST1 sequence (E) or 15-mer peptides in minipool37-39 (F). Data shown are representative of two independent experiments. Data represents mean ± SEM. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

DISCUSSION

Elucidation of novel and potential therapeutic targets for treating malignant mesothelioma remains an urgent need in the absence of effective treatments for this aggressive tumor type. Previous studies have described the use of yeast and poxviral vectors to deliver TWIST1 as an immunotherapeutic approach to elicit antigen-specific T cell responses to control breast and prostate cancers in mouse models.[26,27,34] In comparison, this study is the first, to our knowledge, to demonstrate the induction of TWIST1-specific T cells with a DNA vaccine in an immune-competent mesothelioma cancer model. Our results demonstrate that sPD1-TWIST1 vaccination has potential as a therapeutic intervention for mesothelioma immunotherapy because it provides tumor suppression in both s.c. and metastatic mesothelioma challenges that are dependent on TWIST1-specific T cell responses. Importantly, we show that sPD1-TWIST1 vaccination in combination with CTLA-4 immune checkpoint blockade further activates and enhances TWIST1-specific T cells with better cytotoxic activity and long-lasting memory in an immunosuppressive TME, leading to durable tumor regression and survival benefit against the established AB1 mesothelioma and 4T1 breast cancer in mice. Finally, we found that efficacious T cells recognize a highly immunodominant short peptide that is highly conserved across murine and human TWIST1 sequence, thus providing rationale for further optimization of a human PD1-TWIST1 vaccine to maximize its efficacy and minimize potential side-effects.

Our study shows that TWIST1 is required for mesothelioma invasion and metastasis. In numerous tumor models, cancer cells were shown to remain dependent on TWIST1 to sustain proliferation or to promote metastatic spread through EMT induction.[20,21,23] However, only two previous publications, one in abstract form, have reported the possible association between upregulated TWIST1 expression and poor prognosis in mesothelioma.[24,25] The role of elevated TWIST1 expression in mesothelioma remains unexplored. Here, we report the link between TWIST1 expression and clinical outcomes of mesothelioma patients. By KO and overexpression approaches, we further demonstrate that TWIST1 promotes expression of EMT-related molecules and positively regulates mesothelioma cell invasion *in vitro* and metastasis *in vivo*. The promotion of invasion by TWIST1 was detected in two different invasion assays. To study the metastatic potential mediated by TWIST1 expression *in vivo*, we established an experimental metastasis model by i.v. injection of AB1 cells, which results in lung

metastasis and rapid death of animals. We found that silencing TWIST1 nearly abolished the metastatic ability of AB1 mesothelioma, while its overexpression did not further enhance metastasis significantly, implying that maintenance of extremely high TWIST1 expression may not be necessary for mesothelioma when invasion and intravasation are accomplished.[22,23] Interestingly, though to a lesser extent, TWIST1 promotes clonogenic potential and s.c. tumor growth of AB1 mesothelioma. In line with this, previous studies also found that TWIST1 interferes with the p53 tumor suppressor pathway to provide survival advantage for varieties of malignant cells.[20,21] Overall, inhibiting TWIST1 arrests mesothelioma growth and metastasis. We, therefore, support that TWIST1 could serve as a potential antigen for mesothelioma vaccine.

The sPD1-TWIST1 vaccine is immunogenic for eliciting T cell responses. Targeting TAAs, which are self-proteins abnormally expressed by cancer cells, is a common strategy of tumor vaccines. However, this approach faces the problem of thymic deletion of high-affinity T cells, leaving an attenuated low-avidity repertoire. Nevertheless, therapeutic vaccination of differentiation antigens (e.g., tyrosinase-related protein 2 [TRP2]) or cancer testis antigens (e.g., prostate acid phosphatase [PAP]) has been shown to bypass the thymic tolerance and induce tumor regression in cancer patients.[14,35–37] TWIST1 is expressed mostly in murine testis or human placenta, making it a possible cancer antigen candidate for therapeutic vaccines.[26,38] Indeed, two TWIST1-based vaccines, delivered by either yeast or poxviral vectors, have demonstrated the ability to elicit TWIST1-specific $CD8^+$ and $CD4^+$ T cell immune responses without any apparent toxic effect.[26,27,34] However, both vaccination strategies showed limited T cell activation and therapeutic efficacy, suggesting the need for improving vaccine immunogenicity. Here, we adopted two approaches to enhance TWIST1-specific T cell responses and achieve the most effective tumor clearance. One is to employ the sPD1-based vaccination and the other is to combine this vaccine with immune checkpoint inhibitor.

The present study demonstrates that breaking tolerance to TWIST1 with DNA vaccine requires the fusion of TWIST1 antigen to the sPD1. It has been previously reported that sPD1-based vaccination potentiated HIV-1 p24-specific $CD8^+$ T cell responses by enhancing antigen binding and uptake by DCs via the PD1/PD-L interaction.[28] In addition, sPD1-p24 vaccination as a monotherapy elicited potent effector $CD8^+$ T cells to prevent and cure malignant mesothelioma expressing the p24 xenoantigen.[11] Adaption of such strategy to TWIST1 successfully resulted in the induction of TWIST1-specific $CD8^+$ and $CD4^+$ T cells, which was not achieved by the conventional sTWIST1 vaccination. While the vaccine-encoded sPD1-TWIST1 retains the ability to secrete extracellularly for binding to PD-L1/L2, the use of EP for vaccine administration would induce localized inflammation *in vivo* to promote DC recruitment,[28] which might also contribute to the enhancement of vaccine immunogenicity detected. The vaccine-elicited T cells were reactive toward AB1 mesothelioma via recognizing TWIST1 expression, leading to rejection of implanted s.c. AB1 tumors and reduction of lung metastasis. Notably, our data also illustrate the effective generation of memory antitumor T cell responses from sPD1-TWIST1 vaccination. Preferential expansion of the effector memory $CD8^+$ T cell subset with TNF-α production correlates with tumor eradication. Thus, the ability of sPD1-TWIST1 vaccine to enhance antitumor efficacy observed in this study may be partially attributed to the induction of these effector memory $CD8^+$ T cell. In addition, increased vaccine-elicited IFN-γ- and TNF-α-producing $CD4^+$/$CD8^+$ T cells is accompanied by the suppression of the immunosuppressive network involving MDSCs and $Foxp3^+CD4^+$ Treg. All of these attributes may contribute to the generation of anti-TWIST1 immune responses and to the improved prophylactic effects that we observed with sPD1-TWIST1 vaccination.

The combined sPD1-TWIST1 and α-CTLA-4 antibody treatment works synergistically to enhance TWIST1-specific T cell responses and immunotherapeutic efficacy. Until now, the induction of antitumor T cells through vaccination has been met with less clinical success, potentially because the induced immune responses are not potent or broad enough to generate a desirable clinical outcome or the acquisition of immune checkpoint molecules by effector T cells in the TME render them progressively exhausted and unable to exert effector functions.[13,39,40] In addition, CTLA-4 and PD1 blockades as monotherapy only work in a restricted number of patients, and their clinical benefits are most effective in the presence of preexisting tumor-specific T cell responses.[41] Therefore, recent studies have been exploring combination strategies in order to enhance the overall efficacy of these novel treatment strategies. Combinations of cancer vaccines and immune checkpoint modulation have shown promising results in both preclinical models and cancer patients.[31,42–44] Accordingly, since PD1 blockades would disrupt the targeting of sPD1-TWIST1 protein with PD-L interaction for antigen delivery,[28] we hypothesized that sPD1-TWIST1 vaccination induced antitumor T cell responses can be optimized when combined with α-CTLA-4 antibody instead. We found that neither α-CTLA-4 nor sPD1-TWIST1 vaccination as a monotherapy can induce mesothelioma regression. Their combined immunotherapy, however, induces effective and durable $CD8^+$ CTLs for the clearance of mesothelioma. We believe that this dual treatment works through multiple mechanisms of action. On one hand, sPD1-based DC targeting is essential because the non-targeting vaccine, even in the combined immunotherapy setting, fails to induce T cell responses, thus highlighting the unique advantage of sPD1-based vaccination strategy in priming T cell immunity. On the other hand, α-CTLA-4 is essential to revert immunosuppression on T cell priming, which otherwise is nearly abolished in sPD1-TWIST1 vaccination monotherapy. Nevertheless, activation of $CD8^+$ CTLs is critical to the success of the combined immunotherapy observed in both AB1 mesothelioma and 4T1 breast cancer models, although the detailed mechanisms by which they mediate antitumor activity have yet to be elucidated. Our data also show that antigen spreading following initial tumor lysis cross-primes additional tumor-specific T cell responses to expand T cell repertoire that potentially prevent immune escape, which may work hand-in-hand with the vaccine-elicited TWIST1-specific immune responses for tumor eradication in our model. Together, our results reveal the main

limitations of the use of either vaccination or CTLA-4 blockade as monotherapy against malignant mesothelioma. More importantly, we demonstrate the superiority of the combined sPD1-based vaccination and α-CTLA-4 antibody immunotherapy for promoting antitumor immunotherapeutic efficacy.

Efficacious T cells elicited from combined sPD1-TWIST1 vaccination and α-CTLA-4 therapy recognize a highly immunodominant short peptide within TWIST1 antigen, which has not been reported before. Previously, the TWIST1 epitope LYQVLQSDEL was identified to specifically activate murine CTLs against the 4T1 breast cancer, which was published as an abstract form (B. Wang et al., 2006, Am. Assoc. Cancer Res., abstract). This epitope was used in following studies to detect TWIST1-specific T cell responses by assessing IFN-γ production in *ex vivo* culture supernatant after long-term stimulation of T cells, suggesting it is probably a weak inducer of T cell responses.[26,33,34] In contrast, our results demonstrate that T cells responsive to a short peptide within minipool 37–39, other than the epitope LYQVLQSDEL, are dominantly present in the mice cured of AB1 mesothelioma. It is possible that the specificity observed is dependent on PD1-based vaccination approach and tumor type. Nevertheless, our findings provide rationale for further optimization of PD1-based TWIST1 vaccine design.

In summary, immunization with a sPD1-based DNA vaccine encoding TWIST1 induces long-lasting TWIST1-specific T cell responses, inhibits metastasis, and controls mesothelioma growth. Rational combination of sPD1-TWIST1 vaccination and CTLA-4 immune checkpoint modulation promotes TWIST1-specific T cell-mediated tumor rejection. With a broad range of expression across various solid tumor types, this preclinical study will serve as a foundation for clinical studies targeting human TWIST1 antigen in the future.

MATERIALS AND METHODS

Mice

All mice were maintained according to standard operational procedures at HKU Laboratory Animal Unit (LAU), and all procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of HKU (license #4249-17). 6- to 8-week-old female BALB/c and C.B-17/Icr-scid (SCID) mice were used.

Cell Lines and Culture Conditions

The AB1 cell line, purchased from the European Collection of Cell Cultures, and 4T1 cell line, a kind grift from Prof. Jian-Dong Huang (School of Biomedical Science, HKU), were maintained in complete Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco; supplemented with 10% fetal bovine serum [FBS], 2 mM L-glutamine, and antibiotics). To generate TWIST1 KO tumor cells, HEK293T cells were transfected with the lentiviral expression vector pLentiCRISPR containing Cas9-single guide RNA targeting TWIST1 (TWIST1 sgRNA, 5′-TTGCTCAGGCTGTCGTCGGC-3′) along with pCMV-VSV-G and psPAX2 plasmids, kind gifts from Dr. Kin-Hang Kok (Department of Microbiology, HKU). To generate TWIST1 overexpression tumor cells, the Twist1 gene was cloned into pCDH vector (System Biosciences) and used for transfection of HEK293T cells, together with pPACKH1 lentiviral packaging system (System Biosciences). Virus from supernatants of these transfections were used to transduce AB1 cells followed by puromycin selection. TWIST1 overexpression, KO, and luciferase-expressing cell lines (AB1-Luc) were maintained in complete RPMI supplemented with 1 μg/mL puromycin (Thermo Scientific). T cells and splenocytes were cultured in complete RPMI supplemented with 50 μM 2-mercaptoethanol (Sigma).

DNA Vaccine Constructs

The flexible linker G_4SG_3 (nt 5′-GGTGGTGGTGGTTCAGGAG GAGGA-3′) fragment was introduced at the 5′ end of the full-length mouse Twist1 (nt 1–621). The G_4SG_3-Twist1 fusion cDNA was synthesized by oligonucleotide assembly (GenScript) and was subcloned into pVAX.1 vector. The tissue plasminogen activator signal sequence linked sPD1 (tPA-sPD1) fragment was purified from NheI/EcoRI restriction digestion of our plasmid encoding tPA-sPD1-$p24_{fc}$[28] and subcloned into pVAX.1 vector to generate pVAX.1-tPA-sPD1-Twist1 (Figure 2A). The tPA sequence was amplified with PCR and ligated into the NheI/EcoRI site of pVAX.1 vector to generate pVAX.1-tPA-Twist1. All constructs were routinely sequenced. 100 μg plasmid DNA was used for all the vaccination procedure unless otherwise indicated.

Antibodies

The following antibodies were used for western blotting: anti-TWIST1 (clone Twist2C1a, Abcam), anti-β-actin (clone AC-15, Abcam), and anti-GAPDH (clone EPR16891, Abcam). The following antibodies were purchased from eBioscience and used for flow cytometry: anti-CD11b (clone M1/70), anti-Ly6C (clone HK1.4), anti-Ly6G (clone 1A8-Ly6g), anti-CD3 (clone 17A2), anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), and anti-PD1 (clone J43). The following antibodies were purchased from BioLegend and used for flow cytometry: anti-CD25 (clone 3C7), anti-Foxp3 (clone 150D), anti-CD49b (HMα2), anti-PD-L1 (clone 10F.9G2), anti-PD-L2 (clone TY25), anti-CD44 (clone IM7), anti-CD62L (clone MEL-14), anti-IFN-γ (clone XMG1.2), anti-TNF-α (clone MP6-XT22), and anti-IL-2 (clone JES6-5H4). Cell surface and intracellular immunostaining were performed as previously described.[11] Flow cytometric data analysis was performed using the FlowJo software (Tree Star, v10). Anti-CTLA-4 (clone 9D9) and control mouse immunoglobulin G_{2b} (IgG_{2b}) antibodies were purchased from BioXcell.

Tumor Models

Tumor cells were harvested and single cell suspensions in 100 μL PBS were injected s.c. into right hind flank (for AB1 model) or into the second mammary gland (for 4T1 model) of BALB/c mice. Tumor volumes were measured by caliper (tumor volume = 1/2(length × $width^2$)). Luciferase-expressing tumors were measured with IVIS spectrum (PerkinElmer) and presented as number of photons per second per square centimeter per steradian (photons/s/cm^2/sr) within regions of interest (ROI) using Living Image software (version 4.0, PerkinElmer), as previously described.[11,32] In the AB1 experimental metastasis model, 1×10^6 AB1 cells were injected into the tail vein

of BALB/c mice, and the colonization of AB1 cells in the lung were determined by noninvasive bioluminescence imaging and H&E staining at the endpoint. AB1-Luc rechallenge was performed 60 days after primary tumor ablation on the opposite flank of animals. In the 4T1 spontaneous metastasis model, metastasis of 4T1 tumor cells into the lung are examined with a standard colonogenic assay at the endpoint.[45] Specimens were fixed in zinc formalin fixative (Sigma) and then embedded in paraffin blocks for H&E staining. Metastatic area was defined as the percentage of lung area occupied by metastatic tumor, measured by ImageJ.

Quantitative Reverse Transcriptase PCR

Total cell RNA was extracted with an RNeasy kit (QIAGEN), and cDNA generated by SuperScript III first-strand kit (Thermo Scientific). Then PCR was performed using the following primers with PrimeStar HS DNA polymerase (Takara): Twist1, 5′-AGCTACGCCT TCTCCGTCTG-3′, 5′-CTCCTTCTCTGGAAACAATGACA-3′; vimentin, 5′-TGACCTCTCTGAGGCTGCCAACC-3′, 5′-TTCCATCT CACGCATCTGGCGCTC-3′; N-cadherin, 5′-AAAGAGCGCCAAG CCAAGCAGC-3′, 5′-TGCGGATCGGACTGGGTACTGT G-3′; E-cadherin, 5′-ACACCGATG GTGAGGGTACACAGG-3′, 5′-GCC GCCACACACAGC ATAGTCTC-3′; Fsp1, 5′-CCTGTCCTGCATT GCCATGAT-3′, 5′-CCCACTGGCAAACTACA CCC-3′; Zeb1, 5′-GATTCCCCAAGTGGCATATACA-3′, 5′-TGGAGACTCCTTC TGAGCTA GTG-3′; occludin, 5′-TGCTAAGGCAGTTTTGGCTAA GTCT-3′, 5′-AAAAACAGTGGTGG GGAACATG-3′; actin, 5′-GG CATGGGTCAGAAGGATT-3′, 5′-GGGGTGTTGAAGGTCT CAA A-3′; Gapdh, 5′-GGTCCTCAGTGTAGCCCAAG-3′, 5′-AATGTGT CCGTCGTGGATCT-3′.

In Vitro Tumor Cell-Based Assays

AB1 cells at a density of 0.5×10^4 cells per well were plated into 96-well plates in complete RPMI medium for proliferation assay, with MTS cell viability assay (Promega) performed at 0, 24, 48, and 72 h according to the manufacturer's instructions. A colony-formation assay was performed in 6-well plates with an initial cell density of 500 AB1 cells per well in complete RPMI medium and colonies were stained with crystal violet (0.5 w/v) 9 days later, according to a standard protocol.[46] For the monolayer wound-healing assay, 1×10^6 cells were plated into 6-well plates 1 day before scratching by a plastic tip (1 mm). After cell washing to remove debris, cells were cultured in complete RPMI medium to monitor wound-healing over time. For the cell-invasion assay, AB1 cells were starved overnight in serum-free RPMI medium. 30 μL thawed Matrigel (BD Biosciences) was used to coat each invasion chamber (Transwell, BD Biosciences) equipped with an 8-μm pore size Micropore filter. The chambers were then incubated at 37°C for 30 min and rinsed gently with serum-free RPMI. In the meantime, AB1 cells were harvested after trypsinization and washed once with serum-free RPMI medium. Then 250 μL AB1 cells at a density of 1×10^6 cells/mL in serum-free RPMI was added to the upper chamber. 500 μL RPMI was added to the bottom chamber with 10% FBS as chemoattractant. After incubation at 37°C for 24 h, the Matrigel on the filter was removed with a cotton swab. After crystal violet staining, the membrane was washed several times with PBS before images were taken. Then cell stain was dissolved with extraction buffer for 30 min at room temperature, and absorbance was read with a microplate reader at 560 nm.

Ex Vivo Cell Preparation

Splenocytes were isolated as previously described.[11,32] Tumors were cut into pieces and digested with 1 mg/mL collagenase IV (Sigma) and 0.5 U/mL DNase I (Roche) for 1.5–2.0 h at 37°C. Cells were passed through a 70-μm strainer and then subjected to 40%/80% Percoll gradient (Sigma). Leukocytes at the interphase were recovered after centrifugation at $800 \times g$ for 20 min. T cells, including $CD3^+$, $CD4^+$, and $CD8^+$ T cells, were isolated by an untouched T cell isolation kit (Miltenyi Biotech).

ELISpot, Cytokine Production Assay, and T Cell Cytotoxicity Assay

IFN-γ-producing T cells in isolated splenocytes was assessed by ELISpot assay.[11,32] A mouse TWIST1 peptide library (of 49 peptides generated as 15-mers overlapping by 11 amino acids), gp70-AH1 (SPSYVYHQF), and ovalbumin ($OVA_{257-264}$) were synthesized by GL Biochem (Shanghai). Cytokine concentrations in co-culture supernatant were measured by LEGENDplex T helper cytokine panel (BioLegend). Cytotoxic effect of purified T cells against AB1 cells was determined using a nonradioactive cytotoxicity assay (Promega) according to the manufacturer's instructions.

BMDC-T Cell Co-culture

Following a standard protocol,[32] BMDCs were generated *ex vivo* from isolated bone marrow cells of BALB/c mice in the presence of murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. BMDCs were seeded into 96-well U-bottom plate at 2×10^4 cells per well in the presence of 10 μg/mL TWIST1 or OVA peptide and incubated at 37°C for 1 h, followed by exposure to 1, 10, or 100 μg/mL anti-CTLA-4 antibody (clone 9D9) at 37°C for 1 h. Then, CFSE-labeled $CD3^+$ T cells (1×10^5) were co-cultured with antigen-pulsed BMDCs for 6 days before analysis by flow cytometry. Half of the culture medium was replaced every 2 days, and IL-2 (10 ng/mL) was added into the co-culture at day 4.

Statistical Analyses

All data are presented as mean ± SEM. Information on the study outline, sample size, and statistical analysis is shown in the main text, figures, and figure legends. Significance of mean differences was determined using non-parametric Mann-Whitney U-tests or Wilcoxon matched-pairs tests for unpaired and paired analysis, respectively, to compare datasets. Two-way ANOVA was used to compare mouse tumor volume data among different groups. Survival data was plotted on Kaplan-Meier survival curve, and the log-rank (Mantel-Cox) test was performed to analyze differences in GraphPad Prism 7 software. In all statistical analyses, $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omto.2020.01.009.

AUTHOR CONTRIBUTIONS

Z.T., K.M., and Z.C. conceived the study. Z.T., M.S.C., C.W.Y., and Z.C. designed/performed the experiments and analyzed the data. Z.T., Y.C.W., and Z.C. wrote the manuscript. Y.C.W., H.H., and K.M. provided resources and key experience.

CONFLICTS OF INTEREST

The authors declare they have no competing interests.

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