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Vaccines Targeting Tumor Blood Vessel Antigens Promote CD8+ T Cell-Dependent Tumor Eradication or Dormancy in HLA-A2 Transgenic Mice

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We have recently shown that effective cytokine gene therapy of solid tumors in HLA-A2 transgenic (HHD) mice lacking murine MHC class I molecule expression results in the generation of HLA-A2–restricted CD8+ T effector cells selectively recognizing tumor blood vessel-associated pericytes and/or vascular endothelial cells. Using an HHD model in which HLA-A2neg tumor (MC38 colon carcinoma or B16 melanoma) cells are not recognized by the CD8+ T cell repertoire, we now show that vaccines on the basis of tumors or policing a state of (occult) tumor dormancy. Vaccine efficacy was not observed if (HLA-A2neg) wild-type C57BL/6 mice were instead used as recipient animals. In the HHD model, effective vaccination resulted in profound infiltration of tumor lesions by CD8+ (but not CD4+) T cells, in a coordinate reduction of CD31+ blood vessels in the tumor microenvironment, and in the “spreading” of CD8+ T cell responses to alternate TBVA that were not intrinsic to the vaccine. Protective Tc1-mediated immunity was durable and directly recognized pericytes and/or vascular endothelial cells flow-sorted from tumor tissue but not from tumor-uninvolved normal kidneys harvested from these same animals. Strikingly, the depletion of CD8+, but not CD4+, T cells at late time points after effective therapy frequently resulted in the recurrence of disease at the site of the regressed primary lesion. This suggests that the vaccine-induced anti-TBVA T cell repertoire can mediate the clinically preferred outcomes of either effectively eradicating tumors or policing a state of (occult) tumor dormancy. The Journal of Immunology, 2012, 188: 1782–1788.

Cancer vaccines based on tumor-associated Ags (TAA) have been extensively evaluated in both translational models and in the clinic. Although by most accounts TAA-based vaccines have been found to be immunogenic in promoting increased frequencies of Ag-specific T cell responses in a large proportion of treated patients, they have only rarely proven curative.

Abbreviations used in this article: APM, Ag-processing/presentation machinery; DC, dendritic cell; DLK1, delta-like kinase 1; HBID, hemoglobin-β; HHD, HLA-A2 transgenic mice; NRP, neuropilin; PDGFRβ, platelet-derived growth factor β; PSMA, prostate-specific membrane Ag; RGS5, regulator of G protein signaling 5; TAA, tumor-associated Ag; TBVA, tumor blood vessel Ag; TEM1, tumor endothelial marker 1 (CD34); Tg, transgenic; TME, tumor microenvironment; VEC, vascular endothelial cell; VEGFR, vascular endothelial growth factor receptor.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12$16.00 (1–3). This limitation in efficacy may relate, at least in part, to the heterogeneity of cancer cells found within a given tumor lesion, particularly with regard to subpopulation “immunophenotypes” (4–6). Indeed, many times patients that have exhibited objective clinical responses to immunomodulatory therapies ultimately progress with tumors characterized by defects in their Ag-processing/presentation machinery (APM) and altered immunophenotypes (4, 7, 8).

A theoretical means by which to promote antitumor immunity, while coordinately circumventing the (immuno)phenotypic “instability” of cancer cells themselves, involves the development of vaccines eliciting T cells capable of selectively targeting tumor-associated stromal cells, such as (myo)fibroblasts, vascular pericytes, and vascular endothelial cells (VEC) (9–20). Interestingly, prophylactic peptide-based and/or recombinant vaccines, based on tumor blood vessel Ag (TBVA) such as endoglin (CD105), NG2, platelet-derived growth factor β (PDGFRβ), vascular endothelial growth factor receptor (VEGFR)1, or VEGFR2, have been previously reported to provide partial protection against challenge with tumor cell lines that fail to express these Ags, presumably on the basis of T cell-mediated antiangiogenic activity in the tumor microenvironment (TME) (9–11, 21–27). However, when applied therapeutically, such vaccines have only slowed the progressive growth of established tumors and modestly extended the overall survival period of treated mice (11, 24, 27).

In our recent paper (28), we reported that IL-12 cytokine gene therapy of established HLA-A2neg B16 melanomas growing in HLA-A2 transgenic (Tg) mice results in CD8+ T cell-mediated protective immunity directed against host HLA-A2+ stromal cells within the TME. We now show that therapeutic vaccination of HLA-A2 transgenic mouse (HHD) mice with TBVA-derived
peptides defined in this previous study results in CD8+ T cell-dependent regression of colon carcinoma and melanoma and long-term protection against disease relapse.

Materials and Methods

Mice

HHD mice are Dβ × β2-microglobulin null Tg for the modified HLA-A*0201 human β2-microglobulin single chain (HHD) gene; Ref. 29 and exhibit CD8+ T cell responses that recapitulate those observed in HLA-A2+ human donors (28–30). C57BL/6 wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female 6- to 8-wk-old mice were used in all experiments and were handled in accordance with an Institutional Animal Care and Use Committee-approved protocol.

Cell lines

MC38, a methylcholanthrene-induced (HLA-A2neg) murine colon carcinoma cell line, and B16, an HLA-A2neg melanoma cell line (syngeneic to the H-2b background of HHD mice), have been described previously (31, 32). The T2 cell line is a TAP-deficient T cell/B cell hybridoma that constitutively expresses HLA-A2 (33). All cell lines were free of mycoplasma contamination.

Peptides

All peptides were synthesized using 9-fluorenylmethoxy carbonyl (Fmoc) chemistry by the University of Pittsburgh Cancer Institute’s Peptide Synthesis Facility (a Shared Resource). Peptides were >96% pure based on HPLC profile and mass spectrometric analysis performed by the University of Pittsburgh Cancer Institute’s Protein Sequencing Facility (a Shared Resource).

Production of murine bone marrow-derived DCs and DC.IL12

DC were generated from bone marrow precursors isolated from the tibiae/femurs of HHD mice, as described previously (28). The Ad.mIL-12p70 and Ad.d5 (empty) recombinant adenoviral vectors were produced and provided by the University of Pittsburgh Cancer Institute’s Vector Core Facility (a Shared Resource), as reported previously (34). Fifteen million (day 5 cultured) DCs were infected at multiplicity of infection = 50 with Ad.mIL-12p70 or the control, empty vector Ad.d5. Although control DC produced <62.5 pg IL-12p70/ml/48 h/106 cells, DC.IL12 cells produced 1–10 ng IL-12p70/ml/48 h/106 cells (34).

Vaccine experiments

For prophylactic experiments, HHD mice were immunized s.c. on the right flank with 100 μl PBS or PBS containing 106 syngenic DC.IL12 cells that had been untreated or prepulsed for 4 h at 37°C with 10 μg anti-class I mAb. Immunizations occurred on days −14 and −7, with mice subsequently receiving injections of MC38 (2 × 106) tumor cells in the left flank on day 0. In all cases, treatment groups contained five mice per cohort. For assessment of tumor cellular composition in repeat experiments, MC38 tumors were isolated by surgical resection 10 d after tumor inoculation and prepared for fluorescence imaging, as described below. For therapeutic experiments, MC38 (2 × 106) or B16 melanoma cells (1 × 106) were injected s.c. in the right flank and allowed to establish/progress for 7 d, at which time, the mice were randomized into cohorts of five mice each, with each group exhibiting an approximate mean tumor size of 50–75 mm2. Mice were then untreated or treated with control, syngenic DC.DCIL12 or DC.IL12 (106 cells injected s.c. in the left flank on days 7 and 14) pulsed with synthetic TBVAX peptides. In some experiments, as indicated, in vivo Ab depletions (on days 6, 13, and 20 after tumor inoculation to assess early involvement or on days 60 and 67 or 180 and 187 to assess late involvement) of protective CD4+ T cells or CD8+ T cells were performed and monitored as described previously (28). In all cases, tumor size (area) was monitored every 3–4 d and is reported as mean ± SD in square millimeters.

Evaluation of specific CD8+ T cell responses in HHD mice

MACS (Miltenyi Biotec) CD8+ splenocytes were harvested (from three mice per group) 7 d after the second round of DC-based vaccination (i.e., day 21 after tumor inoculation) and analyzed for reactivity against unpulsed T2 cells, TBVAX peptide-pulsed T2 cells, or day 19 (flow-sorted) B16-derived PDGFRβ+CD31+H-2Kb neg pericytes or PDGFRβ+CD31+H-2Kb neg VECCiso as described previously (28). Where indicated, 10 μg anti–HLA-A2 mAb BB7.2 or control anti-class II mAb L243 (both from American Type Culture Collection, Manassas, VA) were added to replicate coculture wells. After 48 h, supernatants were analyzed for murine IFN-γ content by specific ELISA (lower detection limit = 31.3 pg/ml; BD Biosciences). Data are reported as the mean ± SD of triplicate determinations.

RT-PCR

RT-PCR was performed using primer pairs as described previously (28).

Fluorescence imaging of tumor sections

Tumor tissue samples were prepared and 6-μm sections prepared as reported previously (34). The following Abs were used: (for T cell analyses), rabbit anti-mouse NG2 (Millipore, Bedford, MA) and Alexa488-conjugated anti-CD4 or -CD8 Abs or matching isotype controls (all from BD Biosciences); (for vascular analyses), rat anti-mouse CD31 (BD Biosciences) and rabbit anti-mouse NG2 (Millipore) Abs; and (for TBVAX), rat anti-mouse CD31 (BD Biosciences) and guinea pig anti-mouse NG2 (provided by Dr. B. Stallow, Burnham Institute for Medical Research, La Jolla, CA) Abs, along with anti-TBVAX as described previously (28). Imaging was performed using an Olympus Fluoview 500 Confocal microscope (Olympus America, Center Valley, PA).

Cutaneous wound healing assays

Wound-healing analyses were performed in HHD mice as described by Maciag et al. (22).

Statistical analysis

Two-tailed Student’s t test or two-way ANOVA were used to test overall differences between groups (StatMate III; ATMS, Tokyo, Japan) with p < 0.05 considered significant.

Results

Vaccines incorporating peptide epitopes derived from TBVAX are immunogenic and protect HHD mice against HLA-A2neg MC38 tumor challenge

To assess the immunogenicity of TBVAX-derived (HLA-A2-presented and evolutionarily conserved 9- or 10-mer; Ref. 28) peptides, female HLA-A2 Tg (HHD; lacking murine H-2b class I molecules) mice were vaccinated twice on a weekly schedule with 106 peptide-pulsed (HHD) DC.IL12 cells. DC.IL12 cells were chosen as a standard “adjuvant” for these studies, based on our previous work demonstrating the capacity of this vehicle to effectively promote robust T helper-independent priming of CD8+ T cells when using only class I-presented peptides in the vaccine formulation (34). One week after the booster immunization, CD8+ splenocytes were isolated and analyzed for their ability to secrete IFN-γ in response to peptide-pulsed HLA-A2+ T cells in vitro. As shown in Fig. 1A, the majority (17 of 20; p < 0.05 versus T cells stimulated with DC only) of TBVAX-derived peptides anal-

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nogenicity and efficacy were not always correlated with one an-
other in the MC38 prophylaxis model (Supplemental Fig. 2), a
finding in accordance with reports for peptide-based vaccines in
human clinical trials (1–3).

Protective vaccines incorporating TBVA peptides promote
enhanced infiltration of the TME by CD8+ T cells in
association with an inhibition of tumor vascularity

Because a cohort of the protective vaccines allowed for a transient
period of tumor growth (prior to ultimate tumor regression), we
isolated MC38 tumor lesions from all cohorts of animals with evi-
dence of disease on day 14 (after tumor inoculation) and performed
immunofluorescence microscopy on tumor sections. We observed
that although control (untreated or vaccinated with DC.IL12/no
peptide) mice contained few CD8+ T cells in the TME, the major-
ity of the peptide vaccinated cohorts exhibited a variable but sig-
nificantly elevated number of CD8+ TILs (Fig. 2A, 2B). In marked
contrast, CD4+ T cell infiltration in the TME was sparse, and the data
were indistinguishable when comparing control versus vaccinated
mice (data not shown). An analysis of vascular structures in these
tumors revealed that mice prevaccinated with peptides derived from
the TBVA EphA2, RG55, or TEM1 had the greatest degree of
suppression in CD31+ vessel counts in the MC38 TME, with
somewhat less pronounced effects also noted for groups vaccinated
against HBB or VEGFR2 ($p < 0.05$ versus untreated mice or
mice

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**FIGURE 1.** Induction of specific/protective CD8+ T cells reactive
against TBVA as a consequence of DC/peptide-based vaccination. A, HHD
mice (five animals per cohort) were vaccinated twice (day 14 or 7) s.c. with
PBS or with isologous DC.IL12 pulsed with PBS or synthetic peptides
(Supplemental Table I) derived from the indicated TBVA. In cases where
more than one peptide was identified for a given target Ag, an equimolar
pool of the indicated peptides (each 10 μM) was pulsed onto DC.IL12 and
used for vaccination in the relevant cohort. One week after the second
immunization, spleens were harvested and splenic CD8+ T cells isolated
using MACS beads (Miltenyi Biotec). T cells were stimulated in vitro for
48 h using the HLA-A2+ T2 cell line pulsed with relevant TBVA versus
irrelevant HIV-nef190-198 (AFHHV AREL; Ref. 24) peptides. Cell-free
supernatants were then recovered and IFN-γ levels were determined by
ELISA. Data are reported as mean ± SD for triplicate ELISA determina-
tions, and are representative of three independent experiments per-
formed. *$p < 0.05$ versus HIV-nef control peptide responses. B, HHD mice
were vaccinated twice (days –14 and –7; right flank) s.c. with PBS or
with isologous DC.IL12 pulsed with PBS or synthetic TBVA peptides as
indicated in A. In cases where more than one peptide is identified for
a given target Ag, an equimolar pool of the indicated peptides (each 10
μM) was pulsed onto DC.IL12 and used for vaccination in the relevant
cohort. One week after the booster vaccine (i.e., day 0), animals were
challenged s.c. on their left flank with $2 \times 10^6$ MC38 colon carcinoma
cells. Tumor growth was then monitored every 3–4 d through day 24. All
data represent mean tumor area (in mm²) ± SD determined from five mice/
cohort, and are representative of three independent experiments per-
formed. *$p < 0.05$ versus DC only on the indicated days.

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**FIGURE 2.** MC38 tumors in mice prevaccinated with TBVA-derived
peptides exhibit differential infiltration by CD8+ T cells and alterations in
vascular density. On day 14 MC38 tumors were harvested from HHD mice
that had been vaccinated as outlined in Fig. 1B with the indicated peptides
(or control PBS or DC.IL12 alone = no peptide). A, Six-micrometer tissue
sections were costained with anti-CD8 (green) and anti-NG2 (red) Abs and
imaged by fluorescence microscopy. Blue signal = nuclear counterstain
using DAPI. Original magnification $\times 40$. B provides a summary of the
mean ± SD number of CD8+ cells per high-power field (HPF) in MC38
tumors isolated from control or vaccinated mice as depicted in A. C, Tissue
sections were costained with anti-CD31 (green) and anti-NG2 (red) Abs
and imaged by fluorescence microscopy. Blue signal = nuclear counter-
stain using DAPI. Original magnification $\times 40$. D, The mean ± SD number
of CD31+ vessels per HPF of MC38 tumors in control or vaccinated mice
are summarized. Representative data are depicted from one of three in-
dependent experiments performed. *$p < 0.05$ versus DC only or untreated
control mice.
vaccinated with DC:IL12/no peptide; Fig. 2C, 2D). Correlative analyses indicated an association between the antitumor efficacy of vaccines and their ability to promote CD8+ T cell infiltration and reduced vascularity in the TME (Supplemental Fig. 2).

Therapeutic vaccines incorporating TBVA-derived peptide epitopes are effective against established HLA-A2neg MC38 colon carcinomas and HLA-A2neg B16 melanomas in HHD mice

Given the robust antitumor activity noted for vaccines based on a subset of TBVA in the prophylactic model, we next studied how well these vaccines would perform as immunotherapies in mice bearing established day 7 s.c. MC38 or B16 tumors. In the MC38 model, we treated HHD mice with DC:IL12 cells pulsed with (an equimolar mixture of) peptides derived from an TBVA shown most capable of regulating tumor growth under prophylactic conditions (Fig. 1B) and exhibiting the highest degree of immunogenicity, based on data provided in Fig. 1A (i.e., DLK1326–334, EphA2883–891, HBB31–39, NRP1469–877, PDGFRβ890–898, RGS55–13, and TEM1691–700). As shown in Fig. 3A, the combination peptide vaccine effectively promoted the regression of established MC38 tumors. Furthermore, on the basis of the Ab–depletion analyses, therapeutic benefit was largely due to the action of CD8+, but not CD4+, T cells (Fig. 3A).

Therapeutic vaccines applied to mice bearing B16 melanomas were also effective in suppressing tumor growth if: 1) the vaccine incorporated peptides derived from the stromal Ags DLK1, EphA2,
HLA-A2+ T2 cells (control or pulsed with the indicated peptides) as described in the preliminary MC38 protection model (Fig. 1B).

**HHD mice cured of B16 tumors by TBVA peptide-based therapeutic vaccines exhibit extended survival and durable Tc1 responses against tumor-associated pericytes and/or VEC and spreading in anti-TBVA CD8+ T cell repertoire**

We followed mice treated in Fig. 3B through 60 d after tumor inoculation and observed significant survival benefits if the animals had been treated with vaccines containing peptides derived from the TBVA DLK-1, EphA2, HBB, NRP1, RGS5, or TEM1 (Fig. 3C, Supplemental Table I). To analyze the status and specificity of Tc1 cells, HHD mice rendered free of B16 melanoma after therapeutic vaccination with DLK or RGS5 peptide-based vaccines were sacrificed 60 d after tumor inoculation. Fresh MACS-isolated spleen CD8+ T cells were then analyzed for reactivity against HLA-A2*PDGFRb*CD31neg pericytes, HLA-A2*PDGFRnegCD31+ VEC, or HLA-A2neg tumor cells flow-sorted from day 19 B16 tumors growing progressively in untreated HHD mice. As shown in Fig. 4, splenic Tc1 cells isolated from mice cured after vaccination with DLK1 peptides recognized tumor-associated pericytes and VEC in an MHC class I-restricted manner. They failed to recognize pericytes or VEC isolated from the tumor-uninvolved kidneys of these same donor animals. These type 1 CD8+ T cells strongly recognized the DLK1 peptides used in the protective vaccine formulation, but also (to a variable degree), a number of additional TBVA-derived peptides that were not included in the therapeutic vaccine. Similarly, B16-bearing HHD mice cured using a vaccine on the basis of the RGS55–13 peptide, demonstrated clear Tc1 recognition of tumor (but not tumor-uninvolved kidney) pericytes, as well as statistically significant response against HLA-A2* T2 cells pulsed with peptides derived from the TBVA DLK1, EphA2, NG2, NRP1, PSMA, RGS5, or TEM1 (Fig. 4).

**HHD mice cured of B16 tumors by TBVA peptide-based therapeutic vaccines either exhibit true “molecular cures” or a state of CD8+ T cell-mediated tumor dormancy**

Despite the high frequency of complete tumor regressors as a consequence of treating B16-bearing HHD mice with TBVA peptide-based vaccines, it was conceivable that TBVA-targeting T cells limit tumor expansion yielding occult disease rather than the complete eradication of cancer cells (i.e., “molecular” cure). To assess this possibility, effectively treated HHD mice with no evidence of (macroscopic) disease were depleted of CD8+ or CD4+ T cells on days 60 and 67 or 180 and 187 by injection of specific Abs in vivo. As shown in Fig. 5, depletion of CD8+ T cells, but not CD4+ T cells, resulted in the re-establishment of melanoma growth at sites of the primary tumor placement in seven of nine (i.e., 78% for depletions on day 60 or 67) and three of eight (i.e., 38% for depletions on day 180 or 187) cases, respectively. Interestingly, two of nine (22%) mice in the day 60 or 67 CD8+ T cell-depleted group exhibited transient tumor expansion and regression and then the reappearance and size of melanomas every 4–7 d. The number of animals evaluated per cohort is indicated within a given panel, with each line representing longitudinal data from a given animal. Data are cumulative from three experiments performed.

**FIGURE 4.** HHD mice cured of B16 melanoma by treatment with DC. IL12/peptide vaccination exhibit polispecific anti-TBVA Tc1 responses. HHD mice bearing established day 7 B16 melanomas were therapeutically vaccinated with peptides derived from the TBVA DLK-1 or RGS5 as described in Fig. 3B. Tumors regressed completely over the next 2 wk. Sixty days after tumor inoculation, CD8+ T cells were MACS isolated from the spleens of these animals and evaluated for IFN-γ production (by ELISA) in response to pericytes and VEC (flow-sorted from day 19 untreated B16 tumors or tumor-uninvolved kidneys of B16-bearing HHD mice) as well as HLA-A2* T2 cells (control or pulsed with the indicated peptides) as described in Materials and Methods. *p < 0.05 versus anti-class I mAb blockade (when evaluating responses against pericytes, VEC, or B16 tumor cells) or T2 cells only (when evaluating antipeptide responses). Data are representative of responses observed in three independent experiments.

**FIGURE 5.** In vivo depletion of CD8+, but not CD4+, T cells from a cohort of HHD mice effectively treated with TBVA peptide-based vaccines results in recurrence of disease at the site of primary tumor inoculation. HHD mice harboring established s.c. B16 melanomas received vaccines consisting of syngenic DC.IL12 pulsed with a mixture of TBVA peptides on days 7 and 14 (after tumor inoculation) as outlined in Fig. 3A, resulting in tumor regression in 100% of treated animals. On days 60 and 67 or days 180 and 187 (after tumor inoculation), mice were depleted of CD4+ or CD8+ T cells by i.p. injection with specific Abs as described in Materials and Methods. Control animals received i.p. injections of isotype control Abs. Specific T cell subset depletions were confirmed by flow cytometry analyses performed on peripheral blood obtained by tail puncture (data not shown). Animals were then monitored for the reappearance and size of melanomas every 4–7 d. The number of animals evaluated per cohort is indicated within a given panel, with each line representing longitudinal data from a given animal. Data are cumulative from three experiments performed.
then “spontaneous” regression over a period of weeks to months (Fig. 5), presumably as TBVA-tumor-specific CD8+ T effector cells were recovered in these animals. We also noted that at the time of primary disease recurrence in CD8+ T cell-depleted animals, melanomas did not present in distant cutaneous sites and that metastases were not detected in the lung, liver, or brain based on a histopathology examination of resected tissues (data not shown).

Discussion

One major finding of the current report is that vaccines based on a subset of TBVA-derived peptides elicit protective/therapeutic immunity against HLA-A2B8 (MC38 or B16) transplantable tumors in HHD mice because of the apparent CD8+ T cell targeting of HLA-A2+ pericytes or VEC in the TME. Once protective anti-TBVA immunity was established as a consequence of specific immunization, vaccinated animals exhibited durable protection against challenge with tumors of divergent histology (Supplemental Fig. 3). Similar peptide-based vaccines applied to CD8-depleted HHD mice or HLA-A2B8 recipient (C57BL/6) mice failed to yield treatment benefit, arguing for the critical involvement of CD8+ T cells and the need for these effector cells to target HLA-A2+ stromal cells in vivo (Fig. 4, Supplemental Fig. 3). A second major finding is that many apparent complete responders in our therapeutic vaccine models retain occult disease, because CD8+, but not CD4+, T cell depletion of “cured” animals resulted in the rapid recurrence of tumors selectively at the site of the original primary lesion in many cases. Although in most instances, recurrent tumors grew quickly and proved lethal, in some cases (i.e., 2 of 10), tumors grew slowly and subsequently underwent spontaneous regression presumably after the Ab-depleted CD8+ T cell repertoire had recovered. These data suggest that TBVA peptide-based vaccines promote complete eradication of tumors or the establishment of a state of (occult) tumor dormancy over extended periods of time, which is regulated by vaccine-instigated CD8+ T cells.

The exact nature of residual occult disease in treated animals that recur upon CD8+ T cell depletion remains unknown. In our HHD model system, we failed to detect direct tumor cell recognition by therapeutic T cells, hence HLA-A2B8 cancer cells would be afforded the possibility of maintaining microscopic nests that were limited in their expansion potential based on the anti(tumor) angiogenic activity of protective Tc1 effector cells as suggested in alternate models of immune-mediated tumor dormancy (35). Alternatively, or additionally, slowly replicating/quiescent tumor cells or tumor-initiating cell populations may persist in low numbers in close proximity to blood vessels within the primary lesion site in the occult disease setting, with such cells undergoing a proliferative switch upon removal of local anti-TBVA CD8+ T cells (36, 37). In such circumstances, combinational vaccines simultaneously targeting multiple TBVA as well as Ags expressed by tumor cells and/or tumor-initiating cells might be expected to yield higher rates of complete cures (14, 38, 39).

Our data suggest that the strongest “clinical” correlates for vaccine efficacy may be the degree of therapeutic type 1 CD8+ T cell infiltration into the TME and the degree to which Tc1 cells regulate the tumor blood supply. This is in keeping with current paradigms for successful immunotherapy outcome, where levels of specific TILs rather than circulating peripheral blood T cells may be predictive of better clinical prognosis (40). As we have previously suggested (41), treatment-associated vascular “normalization” in the TME may directly result from CD8+ T cell-mediated death or functional disruption of VEC or pericytes (that are required to sustain VEC) in vivo. Such antivascular effects may provide a rich source of dead or dying tumor/stromal cells capable of supporting the corollary cross-priming of an evolving protective Tc1 repertoire (41–43). Indeed, we observed that TBVA-based therapeutic vaccines that were capable of inducing tumor clearance resulted in the “spreading” of the protective memory Tc1 repertoire to include specificity against TBVA unrelated to the original vaccine formulation. Our findings are consistent with the general paradigm of “epitope spreading” in the antitumor T cell repertoire as a herald of, or mechanism underlying, superior immunotherapeutic outcome (44–46) and extend this concept to include T cell specificities against TBVA.

Despite theoretical concerns that the anti-TBVA CD8+ T cell response could negatively impact normal tissue blood vessels or the normal process of neoangiogenesis/neovascularization, we failed to detect vaccine induced: 1) T cell responses against normal tissue pericytes or VEC; or 2) delay in the kinetics of skin closure after full thickness wounding (data not shown). Such differential recognition of tumor over non-tumor blood vessel cells by Tc1 effector cells may well relate to higher levels of TBVA expression (and by extension their derivative MHC-presented peptides) by tumor- versus normal tissue-associated pericytes and VEC (Supplemental Fig. 1B) (28), but this could also reflect tissue site-specific variation in blood vessel cell expression of 1) MHC class I APM components, 2) costimulatory/adhesion or coinhibitory molecules, or 3) “repulsion” molecules that inhibit CD8+ T cell–target cell interactions (4–6, 47–49).

In conclusion, our data support the translational use of TBVA-based vaccines and the integration of TBVA targets in immune monitoring strategies applied to patients with solid forms of cancer. In particular, the ability to immunologically target tumor-associated pericytes and VEC via specific vaccination may pave the way for combinational therapy designs integrating antiangiogenic agents (i.e., TKI, VEGF/VEGFR antagonists) that have thus far yielded promising, but frequently transient, objective clinical responses in cancer patients (48–50). In these individuals, tumor blood vessels that become refractive to therapy are characterized by a high-degree of coverage with supportive pericytes (50), potentially making these structures ideal targets for TBVA vaccine-induced, antipericyte Tc1 cells.

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Disclosures
The authors have no financial conflicts of interest.

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