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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 tumor-associated blood vessel Ags (TBVA) elicit protective Tc1-dependent immunity capable of mediating tumor regression or extending overall survival. 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.

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Abbreviations used in this article: APM, Ag-processing/presentation machinery; DC, dendritic cell; DLK1, delta-like kinase 1; HIB1B, hemoglobin-β; HHD, HLA-A2 transgenic mice; NRP, neuropilin; PDGFRβ, platelet-derived growth factor β; PSMA, prostate-specific membrane Ag; RG55, regulator of G protein signaling 5; TAA, tumor-associated Ag; TBVA, tumor blood vessel Ag; TEM1, tumor endothelial marker 1 (CD34/8); Tg, transgenic; TME, tumor microenvironment; VEC, vascular endothelial cell; VEGFR, vascular endothelial growth factor receptor.

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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 B6 × β2-microglobulin null Tg for the modified HLA-A*0201 human β2-microglobulin single chain (HLA 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 (syngenic 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-fluorenylmethoxycarbonyl (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.Δδ (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). Five million (day 5 cultured) DCs were infected at multiplicity of infection = 50 with Ad.mIL-12p70 or the control, empty vector Ad.Δδ. 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 µg PBs or PBS containing 105 syngenic DC.IL12 cells that had been untreated or preprimed for 4 h at 37°C with 10 µM synthetic peptide(s). Immunizations occurred on days 14 and 17, with mice subsequently receiving injections of MC38 (2 × 106) tumor cells in the left flank on day 0. In all cases, treatments groups contained five mice per cohort.

Production of tumor cellular composition in repeat experiments, MC38 tumors were isolated by surgical resection 10 d after tumor inoculation and analyzed for reactivity against HLA-A2neg tumors (after a transient period of establishment) in HHD mice as described previously (28).

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, TBEA peptide-pulsed T2 cells, or day 19 (flow-sorted) B16-derived PDGFRβ CD31+H-2Kbneg CD451 CD11b− pericytes or PDGFRβCD31+H-2KbnegVEC isolated 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 TBEA), rabbit anti-mouse CD31 (BD Biosciences) and guinea pig anti-mouse NG2 (provided by Dr. B. Stallcup, Burnham Institute for Medical Research, La Jolla, CA). Abs, along with anti-TBEA 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 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 TBV are immunogenic and protect HHD mice against HLA-A2neg MC38 tumor challenge

To assess the immunogenicity of TBV-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+ T2 cells in vitro. As shown in Fig. 1A, the majority (17 of 20; p < 0.05 versus T cells stimulated with DC only) of TBV-derived peptides analyzed primed Tc1 responses in vivo that could be detected in vitro.

We noted that the delta-like kinase 1 (DLK1), EphA2, hemo-globin-β (HBB), NG2, neuropilin (NRP1), NRP2, PDGFRβ, prostate-specific membrane Ag (PSMA), regulator of G protein signaling 5 (RGS5), tumor endothelial marker 1 (TEM1), VEGFR1, and VEGFR2 Ags were expressed in situ by blood vessel cells in the MC38 colon carcinoma TME (Supplemental Fig. 1). These findings were similar to our previous observations in the B16 TME (28). This led us to next analyze whether immunization with TBV-derived peptides on days 14 and 17 would protect HHD mice against a subsequent challenge with HLA-A2neg MC38 tumor cells injected s.c. on day 0. As depicted in Fig. 1B, vaccines incorporating peptides from the TBV DLK1, EphA2, HBB, NRP1, PDGFRβ, RGS5, or TEM1 were effective in preventing HLA-A2neg MC38 tumor establishment or they resulted in the regression of tumors (after a transient period of establishment) in HHD mice. In contrast, vaccines based on the TBV NG2, NRP2, PSMA, VEGFR1 or VEGFR2 yielded minimal protection (Fig. 1B). On the basis of the data provided in Fig. 1, vaccine immu-
nogenicity and efficacy were not always correlated with one another 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 evidence 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 majority of the peptide vaccinated cohorts exhibited a variable but significantly 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, RGS5, 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

![FIGURE 1.](image1)

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 determinations, and are representative of three independent experiments performed. *$p < 0.05$ 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 performed. *$p < 0.05$ versus DC only on the indicated days.

![FIGURE 2.](image2)

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 ×40. B provides a summary of the mean ± SD number of CD8+ cells per 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 counterstain using DAPI. Original magnification ×40. D provides a summary of 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 independent 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, NRP1869–877, 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,

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** DC.IL12 vaccines containing TBVA-derived peptides are therapeutic against MC38 colon carcinomas and B16 melanomas in HHD mice: requirement for CD8+ T cells and HLA-A2+ host (stromal) cells. A, HHD mice bearing established day 7 s.c. MC38 tumors (right flank) were left untreated, or they were vaccinated in the left flank with control DC.IL12 or DC.IL12 pulsed with an equimolar pool (10 μM each) of the following TBVA-derived peptides: DLK1326–334, EphA2883–891, HBB31–39, NRP1869–877, PDGFRβ890–898, RGS55–13, and TEM1691–700. Identical booster vaccines were provided on day 14 after tumor inoculation. As indicated, two vaccine cohorts were treated with depleting anti-CD4 or anti-CD8 mAbs as outlined in Materials and Methods to evaluate the impact of these T cell subsets on therapy outcome. Tumor growth was monitored every 3–4 d through day 28. B, Female HHD or C57BL/6 (B6) mice were inoculated s.c. in the right flank with 1 × 10^5 B16 (HLA-A2neg) tumor cells. After 7 d, animals were randomized into groups of five mice exhibiting tumor lesions with a mean surface area of 60–75 mm². The mice then received vaccines consisting of isologous control or peptide-pulsed DC.IL12 cells s.c. in the left flank on days 10 and 17 (after tumor inoculation). 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. Tumor size (mean ± SD) was monitored every 3–4 d through day 34. A and B, Mean tumor area ± SD is reported for five animals per cohort. Data are from two independent experiments in each case. *p < 0.05 versus DC only on the indicated days. C, HHD mice bearing s.c. B16 melanomas were treated as described in Fig. 3B and followed through day 60 after tumor inoculation. Data are reported in Kaplan–Meier plots depicting overall percentage of surviving animals over time. *p < 0.02 versus DC only; **p < 0.002 versus DC only (with refined p values for differences between treatment cohorts reported in Supplemental Table I). Data are cumulative for three independent experiments performed.
HLA-A2+ T2 cells (control or pulsed with the indicated peptides) as determined by flow cytometry analyses performed on peripheral blood obtained by tail venipuncture (data not shown). Animals were then monitored for the reappearance of melanomas every 4–7 days. The number of animals per cohort is indicated within a given panel, with each line representing longitudinal data from a given animal. Data are cumulative from three independent experiments.

**FIGURE 4.** HHD mice cured of B16 melanoma by treatment with DC. IL12/pulse vaccination exhibit polyspecific anti-TBV A Tc1 responses. HHD mice bearing established day 7 B16 melanomas were therapeutically vaccinated with peptides derived from the TBVA DLK1, EphA2, HBB, NRP1, RGS5, or TEM1 as described in Fig. 3C. 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 venipuncture (data not shown). Animals were then monitored for the reappearance and size of melanomas every 4–7 days. 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 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 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 of their tumors back to a T cell-mediated state of dormancy rather than true disease recurrence.
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 distal 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-A23/8 (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-A23/8 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-A23/8 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 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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