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CD8+ T Cell Responses Against Hemoglobin- β Prevent Solid Tumor Growth

Hideo Komita¹, Xi Zhao¹, Jennifer L. Taylor¹, Louis J. Sparvero^{2,6}, Andrew A. Amoscato^{2,3,6}, Sean Alber⁴, Simon C. Watkins⁴, Angela D. Pardee⁵, Amy K. Wesa¹, and Walter J. Storkus^{1,5,6}

¹Department of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, USA

²Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, USA

³Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, USA

⁴Departments of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, USA

⁵Department of Immunology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, USA

⁶University of Pittsburgh Cancer Institute, Pittsburgh, PA, 15213, USA

Abstract

Bone marrow-derived dendritic cells (DCs) engineered using recombinant adenovirus to secrete high levels of IL-12p70 dramatically inhibited the growth of established CMS4 sarcomas in BALB/c mice after intratumoral administration. An analysis of splenic CD8+ T cells in regressor mice revealed a strong, complex reactivity pattern against HPLC-resolved peptides isolated by acid elution from single-cell suspensions of surgically-resected CMS4 lesions. Mass spectrometry analyses defined 2 major overlapping peptide species that derive from the murine hemoglobin- β (HBB) protein within the most stimulatory HPLC fractions. Although cultured CMS4 tumor cells failed to express HBB mRNA based on RT-PCR analyses, prophylactic vaccination of BALB/c mice with vaccines containing HBB peptides promoted specific CD8+ T cell responses that protected mice against a subsequent challenge with CMS4, or unrelated syngenic (HBB^{neg}) tumors of divergent histology (sarcoma, carcinomas of the breast or colon). *In situ* imaging suggested that vaccines limit or destabilize tumor-associated vascular structures, potentially by promoting immunity against HBB+ vascular pericytes. Importantly, there were no untoward effects of vaccination with the HBB peptide on peripheral red blood cell (RBC) numbers, RBC hemoglobin content or vascular structures in the brain or eye.

Keywords

Interleukin-12; Gene Therapy; Tumor; Hemoglobin- β ; Pericyte

INTRODUCTION

In a previous study, we demonstrated that intratumoral injection of syngenic dendritic cells (DC) engineered to constitutively secrete interleukin-12p70 (IL-12p70) resulted in the

Correspondence to: Walter J. Storkus Departments of Dermatology and Immunology, W1041.2 Biomedical Sciences Tower, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh PA 15213, USA; Tel: 412-648-9981; Fax: 412-383-5857; e-mail: storkuswj@upmc.edu.

sustained vitality of DC that were competent to promote the cross-priming of therapeutic CD8⁺ T cells *in vivo* (1). Of note, the protective CD8⁺ T cell repertoire appeared to recognize a broad array of tumor MHC-presented peptide epitopes, although the identity of these peptides was not elucidated (1). In the present study, we show that at least one of these epitopes derives from stromal components (i.e. vascular pericytes) within the tumor microenvironment, but not from the tumor itself.

In addition to tumor cells, the tumor cell microenvironment is sustained by numerous requisite stromal components, including blood vessels composed of vascular endothelial cells encased within a matrix of “mural” cells, also known as pericytes (2,3). Pericytes serve to stabilize nascent tubules formed from vascular endothelial cells (VEC; refs. 2-4), with pericyte coverage of vascular structures greatest in the brain and eye, where edema could result in significant pathology (2,5,6). In contrast, pericyte coverage of blood vessels in tumors has been reported to be highly variable, resulting in a tortured, leaky vasculature (7). Notably, in experimental cases where pericyte coverage of vascular bodies falls below a critical threshold of 5-10%, endothelial cells may become susceptible to apoptosis, resulting in increased vascular permeability and hemorrhaging/aneurism (2). This is perhaps best exemplified in mice that fail to express the platelet-derived growth factor receptor- β (PDGFR β), as these animals appear deficient in pericytes, and exhibit aberrant vascularization (8). Interestingly, Reisfeld et al. (9) have recently demonstrated that immunization of wild-type mice with a recombinant DNA vaccine encoding PDGFR β promotes the immune-mediated loss of NG2⁺ pericytes within PDGFR β^{neg} solid tumors. Treated animals resist tumor progression and exhibit prolonged survival (9). Our current results suggest, surprisingly, that the “self” antigen HBB may represent yet another pericyte-associated antigen within the tumor microenvironment, against which immunity can be effectively evoked, thereby limiting or ablating tumor growth *in vivo*.

MATERIALS AND METHODS

Mice

Female 6-8 week old Balb/c mice were purchased from the Jackson laboratory (Bar Harbor, ME) and maintained in micro-isolator cages. Animals were handled under aseptic conditions, per an Institutional Animal Care and Use Committee (IACUC)-approved protocol.

Cell Lines and Culture

CMS4 and MethA are chemically-induced BALB/c (H-2^d) sarcomas that have been described previously (1). The TS/A breast carcinoma and CT26 colon carcinoma, both H-2^d, were purchased from the American Type Culture Collection (ATCC; Manassas, VA). These cell lines were free of mycoplasma contamination and were maintained in CM [RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc., Grand Island, NY) in a humidified incubator at 5% CO₂ tension and 37°C.

In vitro Generation of Bone Marrow (BM)-derived DCs

DC were generated from BM precursors isolated from the tibias/femurs of BALB/c mice, as previously described (1). BM cells were cultured in CM supplemented with 10% heat-inactivated fetal bovine serum, 1000 U/ml recombinant murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) and 1000 U/ml rmIL-4 (Peprotech, NJ) at 37°C in a humidified, 5% CO₂ incubator for up to 7 days.

Viral vectors

The Ad.mIL-12p70 and control Ad.ψ5 (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 (1).

Adenoviral Infection of DCs

Five million (day 5 cultured) DCs were infected at an MOI = 50 with recombinant adenoviruses encoding mouse IL-12p70 (Ad.IL-12) or no cytokine (Ad.ψ5). After 48 h, infected DCs (i.e. DC.IL12 or DC.ψ5, respectively) were harvested and analyzed for their phenotype and function, as previously reported (1). Culture supernatants were collected for measurement of mIL-12p70 production using a species-specific ELISA kit (BD-Biosciences, San Diego, CA), with a lower level of detection of 62.5 pg/ml.

Synthetic Peptides

Peptides HBB₃₃₋₄₁ (LVVYPWTQR), HBB₃₄₋₄₂ (VVYPWTQRY), HBB₃₃₋₄₂ (LVVYPWTQRY), OVA₂₅₇₋₂₆₄ (SIINFEKL) and β-galactosidase₇₆₇₋₇₈₄ (β-gal; TPHPARIGL) were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry by the University of Pittsburgh Cancer Institute's Peptide Synthesis Facility (a Shared Resource). Peptides were >96% pure based on high performance liquid chromatography profile and mass spectrometric analysis performed by the University of Pittsburgh Cancer Institute's Protein Sequencing Facility (a Shared Resource).

Animal Experiments

For therapeutic experiments, BALB/c mice received s.c. injection with 5×10^5 CMS4 cells in the right flank on day 0. On day 7, mice were randomized into cohorts of 5 mice each exhibiting average tumor sizes of approximately 20–30 mm². On days 7 and 14, tumor-bearing mice were treated with intratumoral injections of 1×10^6 adenovirus-infected DCs (DC.ψ5 or DC.IL12) in a total volume of 100 μl PBS. Tumor size was then assessed every 3–4 days and recorded in mm² determined as the product of orthogonal measurements taken using vernier calipers. Data are reported as mean tumor area ± SD.

For prophylactic experiments, BALB/c mice were immunized s.c. on the right flank with 100 μl PBS or 100 μl PBS containing 10^6 syngenic DC.IL12 cells that had been untreated or pre-pulsed for 4h at 37°C with the HBB₃₃₋₄₂ peptide (LVVYPWTQRY), or 10^6 syngenic DC.ψ5 cells pulsed with the HBB peptide. Immunizations occurred on days –14 and –7, with mice subsequently receiving injections of CMS4 (5×10^5), MethA (2.5×10^6), TS/A (10^5) or CT26 (10^5) tumor cells in the left flank on d0. As above for the therapeutic model, mean tumor sizes ± SD were evaluated every 3–4 days. In all cases, treatment groups contained 5 mice per cohort. For analysis of tumor cellular composition in repeat experiments, CMS4 tumors were surgically resected 14 days after tumor inoculation and prepared for fluorescence imaging, as described below. Also in some experiments, vaccinated mice were depleted of CD4+ or CD8+ T cells by i.p. injection of 50 μg of mAb GK1.5 (ATCC) or 100 μg of mAb 53-6.7 (kindly provided by Dr. Zhaoyang Yu, University of Pittsburgh), respectively in 100 μl PBS on days –3, –2 and –1. Confirmation of specific T cell depletion was performed by analyzing splenocytes from treated mice by flow cytometry using FITC-labeled anti-CD4 and anti-CD8 mAbs (both from BD-Biosciences) that were not sterically blocked by the corresponding mAbs used for *in vivo* depletion.

Natural Peptide Isolation

CMS4 tumors were harvested from untreated, control mice on d28 post-tumor inoculation, aseptically minced and digested with DNase, collagenase and hyaluronidase (all reagents

Sigma-Aldrich, St. Louis, MO), as previously described (10). After filtration through 70 micron mesh (BD-Biosciences), viable cells were washed 5 times with PBS by centrifugation. Peptides were acid-eluted using citrate-phosphate buffer (pH 3.3) from this viable cell mixture, desalted and consequently separated on reverse-phase high-performance liquid chromatography (RP-HPLC), as described previously (11). Individual HPLC fractions (800 μ l) were split into 2 duplicate aliquots (400 μ l each) and then lyophilized to a volume of approximately 10 μ l to effectively remove organic solvents (acetonitrile, trifluoroacetic acid (TFA)). One series of aliquots were reconstituted in 100 μ l of PBS and stored at -80°C until use for T cell assays. The alternate series of aliquots was stored at -80°C and reserved for analysis by mass spectrometry.

Mass Spectrometry (MS) Analysis of Peptides

HPLC fractions recognized to the highest degree *in vitro* by CD8⁺ T cell responses were analyzed by MS as previously described (11).

Evaluation of CD8⁺ T Cell Responses in the therapy model

Spleens were harvested from 2 mice/group 7 days after the second intratumoral injection of DCs (i.e. day 21 after tumor inoculation) and splenocytes re-stimulated *in vitro* for 5 days with irradiated (50 Gy) CMS4 cells at a splenocyte:CMS4 ratio of 10:1. Responder CD8⁺ T cells were then isolated using magnetic bead cell sorting (MACS; Miltenyi Biotec, Auburn, CA). Finally, CD8⁺ T cells (10^5 cells) and syngenic d7 cultured DC (10^4 cells/well) and HPLC-fractionated peptides (10 μ l/well) were added to individual wells of a 96-well tissue culture plate. Alternatively in some assays, CMS4 (10^4) or MethA (10^4) tumor cells (in the absence or presence of 10 μ M HBB peptides were used as target cells. After 48h incubation, culture supernatants were collected and analyzed for IFN- γ release using a commercial ELISA (BD-Biosciences) with a lower limit of detection of 31.5 pg/ml. Data are reported as the mean $\pm$ SD of duplicate determinations. In some assays, 10 μ g/well of blocking anti-H-2K^d (31-3-4s; ATCC), anti-H-2D^d (34-4-21s; ATCC) and Anti-H-2L^d (28-14-8; Santa Cruz Biotechnology, Santa Cruz, CA) mAbs were added to replicate wells to discern the class I-restriction element (s) used by responder CD8⁺ T cells.

Evaluation of CD8⁺ T Cell Responses in the vaccine model

Spleens were harvested from (non-tumor bearing) mice/group 7 days after the second, weekly s.c. (right flank) vaccination with either DC:IL12 alone or DC:IL12 + HBB₃₃₋₄₂ peptide. Isolated splenocytes were re-stimulated *in vitro* for 5 days with irradiated, immature H-2^d DC that had been pre-pulsed with the HBB peptide (1 μ M for 3h at 37°C then washed twice with PBS) at a splenocyte:DC ratio of 10:1. Responder cells were then cultured with immature DC pulsed with no peptide, 1 μ M irrelevant peptides (β -gal or OVA) or 1 μ M HBB₃₃₋₄₂ peptide and analyzed for intracellular levels of IFN- γ by flow cytometry using a BD FastImmuneTMCD8 Intracellular IFN- γ Detection Kit (BD-Biosciences), per the manufacturer's protocol.

Peptide-Binding Assays

The HBB₃₃₋₄₁, HBB₃₄₋₄₂ and HBB₃₃₋₄₂ peptides, as well as, the positive control β -galactoside₈₇₆₋₈₈₄ and negative control OVA₂₅₇₋₂₆₄ peptides (12) were analyzed for their ability to stabilize H-2L^d class I complexes expressed by the T2.L^d cell line (kindly provided by Dr. Peter Cresswell, Yale University). Briefly, T2.L^d cells (10^6) were incubate in the absence, or presence, of synthetic peptides (0.01-1 μ M) at 37°C for 4h, prior to being washed in PBS and stained on ice for 30 min. using FITC-conjugated anti-H-2L^d mAb 28-14-8 (Santa Cruz Biotechnology). Stained cells were then fixed in 2% paraformaldehyde in PBS and analyzed using an Epics XL flow cytometer (Beckman Coulter, Inc., Miami, FL).

RT-PCR

Reverse transcriptase -PCR (RT-PCR) was performed using the following primer pairs: mHBB (forward), 5'-TCAGAAACAGACATCATGGTGC-3'; mHBB (reverse), 5'-TAGACAATAGCAGAAAAGGGGC-3'; β -actin (forward), 5'-GGCATCGTGATGGACTCCG-3'; β -actin (reverse), 5'-GCTGGAAGGTGGACAGCGA-3'. Cycling times and temperatures were as follows: initial denaturation at 94°C for 2 min (1 cycle), denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 1 min (30 cycles), final extension at 72°C for 5 min (1 cycle). PCR products were identified by image analysis software for gel documentation (LabWorks Software; UVP, Upland, CA) following electrophoresis on 1.2% agarose gels and staining with ethidium bromide.

Imaging of Tissue Sections

Tumor samples were prepared and sectioned as previously reported (13). All washing steps were performed using wash buffer (WB; 0.5% BSA in PBS, Sigma-Aldrich). For staining, the sections were fixed in 2% paraformaldehyde (Sigma-Aldrich) at RT for 45 min., then incubated with 2% BSA for 45 min., washed, then blocked using goat anti-mouse Fab1 fragments (Jackson ImmunoResearch, West Grove, PA) in WB overnight. For analysis of T cell subsets, sections were incubated with FITC-conjugated anti-CD4 or -CD8 β antibodies or matching isotype controls (all from BD-Biosciences) for 1h. For analysis of the CD31, SMA and HBB markers, tissue was first blocked in donkey serum (Sigma-Aldrich) for 40 min. One set of tissue was then incubated with monoclonal anti-mouse smooth muscle actin (SMA)-Cy3 (Sigma-Aldrich), rat anti-mouse CD31 (Millipore, Bedford, MA) and goat anti-mouse hemoglobin β (Jackson ImmunoResearch, West Grove, PA) for 1h and then washed. Tissue was then treated with donkey anti-goat Ig-Alexa 488 (Invitrogen, Carlsbad, CA) along with donkey anti-rat Ig-Cy5 (Jackson ImmunoResearch) for 1 hr and washed. Another second set of tissue was incubated with goat anti-mouse hemoglobin- β (Jackson ImmunoResearch) and with rabbit anti-mouse NG2 (Millipore) for 1h, washed, and then treated with a combination of donkey anti-goat Ig-Alexa 488 (Invitrogen) and donkey anti-rabbit Ig-Cy3 (Jackson ImmunoResearch). After being cover-slipped, slide images were acquired using an Olympus 500 Scanning Confocal Microscope (Olympus America, Melville, NY). Isotype control and specific antibody images were taken using the same level of exposure on the channel settings. Co-localization of probes was determined using a measure co-localization algorithm in Metamorph Imaging Software (Molecular Devices, Sunnyvale, CA). For the analysis of brain and eye tissues, whole body perfusion was performed using 10 ml of PBS followed by 10 ml of 2% paraformaldehyde (Sigma-Aldrich) through the left ventricle after cutting the posterior vena cava. Tissue was excised and immersed in 2% paraformaldehyde for 2 hours. Fixed tissues were then immersed in 2.3 M sucrose in PBS overnight at 4°C, before being frozen in liquid nitrogen-cooled isopentane and being stored at -80°C until sectioning. In addition to standard H&E staining, sequential tissue sections were analyzed by immunofluorescence microscopy. The CD31 and NG2 markers were co-analyzed using secondary goat anti-rat Ig-Alexa 488 (Invitrogen) and goat anti-rabbit Ig-Cy3 (Jackson ImmunoResearch) antibodies, respectively. For co-analysis of hemoglobin- β and CD163 (primary Ab sc-33560 from Santa Cruz Biotechnology), we used secondary donkey anti-goat Ig-Alexa 488 (Invitrogen) and donkey anti-rabbit Ig-Cy3 (Jackson ImmunoResearch) antibodies, respectively. After being cover-slipped, slide images were acquired using an Olympus BX51 microscope (Olympus America, Melville, NY).

Analysis of Red Blood Cells (RBC)

Red blood cells were isolated by tail venipuncture from control mice or mice vaccinated twice with weekly s.c. injections of PBS or 10⁶ DC.IL12 cells pulsed with the HBB₃₃₋₄₂ peptide in their right flank. Blood was isolated 28 days after the first vaccination. RBCs were quantitated

per ml of blood by hemacytometer count, with hemoglobin content estimated by optical absorbance measurements using the method of Kahn et al. (14).

Statistical analysis

Statistical differences between groups were evaluated using a two-tailed Student's t-test or one-way ANOVA (StatMate III, ATMS Co., Tokyo, Japan) as appropriate, with p-values < 0.05 considered significant.

RESULTS

Intratumoral injection of syngenic DC engineered to secrete mIL12p70 promotes tumor regression and amplification of systemic anti-tumor CD8+ T cells *in vivo*

In order to extend our previous work (1), syngenic H-2^d BM-derived DC were engineered to secrete mIL-12p70 (i.e. DC.IL12) by infection with recombinant adenovirus (rAd). As shown in Fig. 1A, DC.IL12, but not control Ad ψ 5-infected DC or uninfected DC secreted high levels (> 1 ng/ml/10⁶ DC/24h) of IL-12p70. Only DC.IL12 (10⁶ cells) when injected into s.c. established d7 CMS4 sarcoma lesions (with an average size of 20-30 mm²) on days 7 and 14 proved competent to suppress tumor progression (Fig. 1B; p < 0.0001 on all days > 14 vs. DC. ψ 5 or uninfected DC), with complete tumor regression observed in 8/10 treated animals (vs. 0/10 for either control DC treatment cohort).

To determine the nature and diversity of specific CD8+ T cell-mediated immunity in these animals, we resected untreated CMS4 lesions, generated single-cell suspensions and then extracted peptides from MHC complexes expressed by these cells using mild acid extraction (10). These peptides were resolved into 50 fractions using reverse-phase high-performance HPLC (RP-HPLC; Fig. 1C). After lyophilizing these fractions to remove organic solvents, peptides were reconstituted in PBS and aliquots loaded onto syngenic DC to serve as antigen-presenting cells for splenic CD8+ T cells isolated from mice (as in Fig. 1B) 28d post-tumor challenge. An IFN- γ ELISA performed on supernatants isolated from these cultures revealed that only DC.IL12 protected mice displayed pronounced CD8+ T cell recognition of fractionated peptides, with 3 major bioactive peaks identified (Fig. 1D).

Mass spectrometric identification of murine hemoglobin- β (HBB) peptide as a putative "tumor-associated" CD8+ T cell epitope

Given the strongest reactivity of protective CD8+ T cells against peptide(s) within HPLC fractions 19 and 20 (Fig. 1D), we prioritized analyses of these mixtures using a combination of MALDI-TOF mass spectrometry (MS) and microcapillary-LC-ESI-tandem MS. Fraction 19 contained a prevalent peptide species of Mr = 1211.4 as determined by MALDI-TOF MS (Fig. 2A). The Mr = 1211.4 peptide was consequently determined to represent the sequence VVYPWTQRY upon Ar gas-mediated fragmentation into its daughter ion species monitored using microcapillary-LC-ESI tandem mass spectrometry (Fig. 2B). Similarly, the dominant peptide species in fraction 20 exhibited a Mr = 1324.8 (Fig. 2C), yielding the MS/MS-deduced sequence XVYPWTQRY (with X = Isoleucine or Leucine; Fig. 2D). Based on a consequent protein data base search, these overlapping peptide sequences appear to derive from either the murine hemoglobin- β 1 (HBB1) or - β 2 (HBB2) chains (i.e. both chains contain the sequence LVVYPWTQRY).

We subsequently noted that splenic CD8+ T cells isolated from tumor-bearing mice that had been effectively treated with DC.IL12 were i.) predominantly H-2L^d-restricted in their anti-CMS4 tumor reactivity and ii.) unable to recognize the unrelated H-2^d sarcoma MethA, unless this target cell line was pre-pulsed with the LVVYPWTQRY (HBB₃₃₋₄₂) peptide (Fig. 3A). In the latter case, CD8+ T cell reactivity against the presented HBB₃₃₋₄₂ peptide also

appeared to be largely H-2L^d-restricted based on results obtained in antibody blocking experiments (Fig. 3A). In additional experiments, we determined that each of the two 9-mer (i.e. HBB₃₃₋₄₁ and HBB₃₄₋₄₂), as well as, the 10-mer (HBB₃₃₋₄₂), peptides bound to H-2L^d with modest affinity when compared with a known H-2L^d-presented β -galactosidase₈₇₆₋₈₈₄ peptide epitope (Supplemental Fig. 1).

Mice vaccinated against the HBB₃₃₋₄₂ peptide are protected against consequent tumor challenge

To directly assess the tumor relevance of HBB-derived peptides, naïve BALB/c mice were vaccinated with syngenic DC.IL12, DC.IL12 pulsed with the HBB₃₃₋₄₂ peptide (DC.IL12/HBB) or DC. ψ 5 pulsed with the HBB peptide (DC. ψ 5/HBB) on days -14 and -7, prior to challenging these animals on day 0 with either CMS4 sarcomas or unrelated H-2^d tumors; including MethA (sarcoma), TS/A (breast carcinoma) or CT26 (colon carcinoma). We were not only able to demonstrate specific induction of CD8⁺ Tc1 responses in DC.IL12/HBB vaccinated mice vs. control (DC.IL12 alone) vaccinated mice via intracellular staining for IFN- γ (Fig. 3B), but also, showed that only mice immunized with DC.IL12/HBB were protected against tumor establishment/progression (Fig. 4A). Notably, protection was observed not only against CMS4 tumors, but also against s.c. challenge with the unrelated H-2^d sarcoma (MethA), breast carcinoma (TS/A) or colon carcinoma (CT26) cell lines (Fig. 4A). Animals vaccinated with control DC-based vaccines (DC.IL12 alone or DC. ψ 5/HBB) exhibited progressive lesions in all cases. Interestingly, even the DC.IL12/HBB vaccinated group of CMS4-bearing mice developed small palpable lesions on day 7, which subsequently resolved over the following 7-10 days. Extended experiments using specific depleting antibodies *in vivo* confirmed that immune protection was dependent upon CD8⁺ T cells, but not CD4⁺ T cells (Fig. 4B).

***In vivo* targets of anti-HBB CD8⁺ T cells are not tumor cells themselves, but rather tumor-associated stromal cells**

Although these peptides clearly derived from murine origin and could not have been the result of bovine protein contamination (i.e. fetal bovine serum used to culture CMS4 tumors (i.e. bovine HBB chains contain the sequence LVVYPWTQRF; GenBank Accession# NP_776342), we also noted in RT-PCR analyses that cultured CMS4, MethA, TS/A and CT26 tumor cells fail to express message transcripts for HBB (Fig. 4C). Furthermore, mass spectrometry inspection confirmed that HBB-derived sequences were not present in detectable quantities in (acid-eluted) peptides harvested from CMS4 tumor cells grown *in vitro* (data not shown).

These results suggested that either i.) tumor cells turn on HBB transcription as a consequence of growth *in vivo* or ii.) an alternate, non-tumor cell type(s) present within the tumor microenvironment may present HBB-derived peptides and constitute a clinically-relevant target for protective anti-tumor immunity. We attempted to address this using immunohistochemistry in two ways. First, we theorized that the composition of early CMS4 lesions in HBB peptide immunized mice would differ from that of control vaccinated mice, in that the HBB⁺ target cell population might be selectively depleted. Secondly, we directly analyzed control CMS4 (untreated) lesions for expression of HBB protein in an attempt to co-localize it with stromal cell subsets.

In Fig. 5A, we noted that d14 CMS4 lesions in mice pre-immunized with DC.IL12/HBB (as in Fig. 4A) contained higher frequencies of CD8⁺ T cells (Fig. 5Ab) and comparable levels of CD4⁺ T cells (Fig. 5Ad) when compared to control cohorts (Figs. 5Aa,c). We also observed that CMS4 tumors in the DC.IL12/HBB vaccinated cohort of mice contained fewer CD31⁺ vascular structures vs. control tumors (Fig. 5Af vs. Fig. 5Ae; Fig. 5B; $p < 0.0001$) and that the CD31⁺ vessels in the DC.IL12/HBB immunized group exhibited a reduced level of coverage

by SMA+ pericytes vs. controls (Fig. 5Af vs. Fig. 5Ae; Fig. 5C; $p < 0.001$). Since CD31+ vessels can be compromised in the absence of sufficient pericyte coverage (2), this could suggest pericytes as a logical primary immune target for HBB-specific CD8+ T cells in these animals. When performing co-staining analyses, we noted that HBB protein: i.) did not appear to be expressed by CMS4 sarcoma cells *in situ* (Fig. 5Ag,h), ii.) appeared to be expressed by SMA+ pericytes and/or CD31+ VEC (Fig. 5Ag,h), and iii.) was comparatively depleted in the tumors of DC.IL12/HBB vaccinated vs. control vaccinated mice (Fig. 5Ah vs. Fig. 5Ag). Further IHC analyses using progressive d14 CMS4 tumors harvested from control, untreated mice suggested that HBB expression was primarily associated with SMA+ and/or NG2+ pericytes, and less so with CD31+ vascular endothelial cells (Fig. 6A, 6B). Fluorescence confocal microscopy also revealed that many perivascular HBB+ cells within CMS4 tumors co-expressed CD163 (Fig. 6C), a scavenger receptor for the haptoglobin-hemoglobin complex (14).

DC.IL12/HBB peptide-vaccinated mice do not exhibit RBC-associated abnormalities or tissue pathology in the brain or eye

Since the HBB₃₃₋₄₂ peptide used to vaccinate mice represents a normal, “self” protein sequence that is also expressed by red blood cells (RBC), it was entirely possible that HBB-specific CD8+ T cells could mediate autoimmune anemia. However, we did not detect any significant difference in the numbers of RBC/ml of blood or the level of hemoglobin/dl of peripheral blood in DC.IL12/HBB vaccinated animals vs. control (PBS) vaccinated mice (Supplemental Fig. 2).

Although there were no obvious behavioral alterations in DC.IL12/HBB vaccinated mice that would suggest impairment of brain/visual functions, given the importance of pericytes to the integrity of blood vessels in the brain and eye (2,5,6), it was also important to rule out pathologic consequences of HBB-based vaccines targeting these organs. To further corroborate a lack of vascular alterations in the brain and eye, tissues were harvested from control untreated mice or mice vaccinated with DC.IL12 only vs. DC.IL12/HBB. We noted no discernable inflammatory infiltrates or alterations in tissue-associated vasculature (Supplemental Fig. 3) and we failed to observe expression of CD163 on microglial cells (Supplemental Fig. 4), which has been reported to represent a marker of encephalitis (14). Interestingly, unlike the tumor stroma, we were not able to detect expression of perivascular HBB in either brain or eye tissues (Supplemental Fig. 4).

DISCUSSION

In extending our previous evaluation of the effectiveness of intratumoral administration of DC.IL12 in promoting the cross-priming of therapeutic immunity (1), we identified HBB as a tumor microenvironment-associated antigen recognized by T cells. In particular, the HBB₃₃₋₄₂ peptide and its two derivative 9-mer peptides appear capable of binding to H-2L^d (that appears to serve as the dominant restricting allele for protective T cells in the CMS4 model) and could each represent (at least cross-reactive) epitopes for CD8+ T effector cells. Although our attempts to generate stable H-2L^d/HBB peptide tetramers have failed to date, using an intracellular staining assay for IFN- γ production as a single-cell readout of “clonal” T cell reactivity, we have shown that anti-HBB₃₃₋₄₂ CD8+ T cells are enriched (representing up to 5% of all CD8+ T cells; Fig. 3B) in the spleens of DC.IL12/HBB peptide immunized mice vs. control vaccinated mice.

While this is the first report for CD8+ T cell epitope(s) derived from HBB, a recent publication describes the biochemical identification of CD8+ T cell epitopes derived from γ -globin (16). Like HBB in our model, γ -globin is a normal, “self” protein target to which tolerance would be presumed to be in effect. However, in contrast to our findings in which HBB appears to be

expressed by stromal components and not the tumor itself, γ -globin appears to represent an endogenous tumor cell product, with juvenile myelomonocytic leukemia cells being directly recognized by specific CD8+ T cells (16).

Arguably, the most intriguing aspects of the current study are that: 1) HBB, an antigen most strongly affiliated with erythropoiesis (17), may be expressed by cell types within the tumor microenvironment to which specific CD8+ T cells may be targeted in a class I-restricted manner, 2) CD8+ T cells of a “therapeutically-relevant” functional avidity may be evoked against this “self” antigen in the absence of overt autoimmune pathology affecting RBC or normal vasculature in the brain and eye, and 3) DC.IL12/HBB-immunization results in specific immunity that protects against consequent challenge with unrelated HBB^{neg} tumor cell lines (CMS4, MethA, TS/A and CT26) of disparate histologies. Hence, tumor cells need not express HBB themselves in order for T cell-mediated protection to occur. This does not mean that protection occurs in a completely tumor-independent manner, since clearly tumor cells may condition the microenvironment in a way that (uniquely) promotes HBB peptide presentation by stromal cells, such as NG2+ and/or SMA+ pericytes. This hypothesis is further supported by the observed lack of expression of HBB protein by perivascular cells in normal brain/eyes (Supplemental Fig. 4).

While we believe that pericytes are indeed the HBB+ cells targeted by protective CD8+ T cell-mediated immunity in our model, based on the preferential depletion of this cell type in the tumor lesions of mice vaccinated with DC.IL12/HBB. However, we have not formally demonstrated that pericytes actually transcribe and translate HBB mRNA *in situ*. Still, this would not be surprising given reports of unexpected HBB+ cell types, such as LPS-activated macrophages (18) or alveolar type-2 epithelial cells (19). Furthermore, hemoglobin transcription can be turned on under hypoxic conditions (20,21), such as those encountered in the tumor microenvironment, which could affect tumor-associated pericytes (or VEC). A preliminary RT-PCR analysis for HBB mRNA expression in flow-sorted NG2+ vs. CD31+ cells isolated from progressive CMS4 tumors yielded positive signals for both types of cells (Supplemental Fig. 5). However, these sorted cells were only 76-86% pure and this conclusion must remain cautiously interpreted until confirmatory experiments can be performed on even cleaner cell populations.

An alternate or additional possibility is that pericytes may cross-present HBB-derived peptides in H-2L^d complexes, but fail to express HBB mRNA. Pericytes (and VEC) are active in caveolin-1-dependent transcytosis of exogenous proteins (22,23). Hence, it is feasible that serum HBB protein may be captured from the lumen of vascular vessels or as a result of vascular permeability (24), resulting in VEC/pericyte presentation of HBB peptides in our model. HBB uptake could be specifically mediated by CD163 (a hemoglobin scavenger receptor, ref. 25) which we have demonstrated is expressed by tumor-associated perivascular cells. It is also important to note that since the HBB-specific antibody probe that we used in this study does not recognize the immunogenic HBB₃₃₋₄₂ peptide itself (data not shown), it is also formally possible that tumor-associated APC may serve as HBB cross-presenters to tumor-infiltrating effector CD8+ T cells (25,26).

So, why should HBB be considered a logical and relevant “tumor-associated” target antigen? Although conventionally linked with oxygen transport in RBC, hemoglobin mediates a more primordial function in protecting cells against oxidative and nitrosative stress (27,28) and has been shown to be intimately involved in nitric oxide metabolism (28,29). By being produced within, or taken up by, cells within the tumor microenvironment (such as pericytes or VEC), the associated angiogenic/vasculogenic processes might be expected to be stabilized, leading to progressive tumor growth. Interestingly, a C-terminal fragment of HBB has been reported to be specifically increased within head-and-neck squamous cell carcinomas in a disease stage-

dependent manner, which may support the enhanced presence and proteolytic processing of HBB during disease progression (30). This could also suggest that the “unstable” N-terminus of the protein is selectively degraded/processed and more likely to yield MHC class I-presented epitopes (such as the HBB₃₃₋₄₂ peptide defined in this study).

Overall, these results and recent results from others (9,31-34) support the likely clinical safety and efficacy of combinational vaccine approaches targeting the tumor-associated vasculature. Although similar concepts in the targeting of VEC using VEGFR inhibitors (among others) and pericytes using the PDGFR kinase inhibitor ST1571 have resulted in VEC apoptosis, inhibited tumor growth and prolonged survival in orthotopic murine models of ovarian carcinoma (35), specific CD8⁺ T cells prompted by vaccines would be presumed to provide a more stable, durable mode of protection to patients with active disease or those at high-risk of recurrence. Importantly, while we did not observe any acute toxicities in the blood, brain or eyes of DC.IL12/HBB-vaccinated mice, off-target effects could result from such approaches and prospective studies should investigate whether such vaccines inhibit wound-healing or promote any deleterious destabilization of normal vascular structures over extended periods of time.

Finally, it is important to acknowledge that (intratumorally-delivered and vaccine) DC.IL12 but not control DC.ψ5 served as competent “adjuvants” in eliciting anti-HBB CD8⁺ T cells in our model system. This is consistent with prior reports documenting the critical role of IL-12p70 in breaking operational tolerance to self antigens (36,37) and supports the clinical use of DC.IL12 in cancer vaccine formulations targeting the induction of Type-1 T cell responses against non-mutated antigens/epitopes presented by tumor cells and/or stromal cells within the tumor microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES CITED

1. Tatsumi T, Huang J, Gooding WE, et al. Intratumoral delivery of dendritic cells engineered to secrete both interleukin (IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. *Cancer Res* 2003;63:6378–86. [PubMed: 14559827]
2. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 2005;7:452–64. [PubMed: 16212810]
3. Ganss R. Tumor stroma fosters neovascularization by recruitment of progenitor cells into the tumor bed. *J Cell Mol Med* 2006;10:857–65. [PubMed: 17125590]
4. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. *Exp Cell Res* 2006;312:623–9. [PubMed: 16303125]
5. Hammes HP, Lin J, Renner O, et al. Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 2002;51:3107–12. [PubMed: 12351455]
6. Braun A, Xu H, Hu F, et al. Paucity of pericytes in germinal matrix vasculature of premature infants. *J Neurosci* 2007;27:12012–24. [PubMed: 17978043]
7. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. *Curr Opin Genet Dev* 2005;15:102–11. [PubMed: 15661540]
8. Lindahl P, Johansson BR, Leveén P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF- β -deficient mice. *Science* 1997;277:242–5. [PubMed: 9211853]

9. Kaplan CD, Krüger JA, Zhou H, Luo Y, Xiang R, Reisfeld RA. A novel DNA vaccine encoding PDGFR β suppresses growth and dissemination of murine colon, lung and breast carcinoma. *Vaccine* 2006;24:6994–7002. [PubMed: 17050049]
10. Storkus WJ, Zeh HJ 3rd, Maeurer MJ, Salter RD, Lotze MT. Identification of human melanoma peptides recognized by class I restricted tumor infiltrating T lymphocytes. *J Immunol* 1993;151:3719–27. [PubMed: 7690811]
11. Herr W, Ranieri E, Gambotto A, et al. Identification of naturally processed and HLA-presented Epstein-Barr virus peptides recognized by CD4+ or CD8+ T lymphocytes from human blood. *Proc Natl Acad Sci USA* 1999;96:12033–8. [PubMed: 10518571]
12. Ochoa-Garay J, McKinney DM, Kochounian HH, McMillan M. The ability of peptides to induce cytotoxic T cells in vitro does not strongly correlate with their affinity for the H-2Ld molecule: implications for vaccine design and immunotherapy. *Mol Immunol* 1997;34:273–81. [PubMed: 9224969]
13. Berhanu A, Huang J, Alber SM, Watkins SC, Storkus WJ. Combinational rFlt3-ligand and rGM-CSF treatment promotes enhanced tumor infiltration by dendritic cells and anti-tumor CD8+ T cell cross-priming, but is ineffective as a therapy. *Cancer Res* 2006;66:4895–903. [PubMed: 16651446]
14. Kahn SE, Watkins BF, Bernes EW Jr. An evaluation of a spectrophotometric scanning technique for the rapid determination of plasma hemoglobin. *Ann Clin Lab Sci* 1981;11:126–31. [PubMed: 7259087]
15. Borda JT, Alvarez X, Mohan M, Hasegawa A, Bernardino A, Jean S, Aye P, Lackner AA. CD163, a marker of perivascular macrophages, is up-regulated by microglia in simian immunodeficiency virus encephalitis after haptoglobin-hemoglobin complex stimulation and is suggestive of breakdown of the blood-brain barrier. *Am J Pathol* 2008;172:725–37. [PubMed: 18276779]
16. Hirano N, Butler MO, Xia Z, et al. Identification of an immunogenic CD8+ T-cell epitope derived from γ -globin, a putative tumor-associated antigen for juvenile myelomonocytic leukemia. *Blood* 2006;108:2662–8. [PubMed: 16778141]
17. Stamatoyanopoulos G. Control of globin gene expression during development and erythroid differentiation. *Exp Hematol* 2005;33:259–71. [PubMed: 15730849]
18. Liu L, Zeng M, Stamler JS. Hemoglobin induction in mouse macrophages. *Proc Natl Acad Sci USA* 1999;96:6643–7. [PubMed: 10359765]
19. Bhaskaran M, Chen H, Chen Z, Liu L. Hemoglobin is expressed in alveolar epithelial type II cells. *Biochem Biophys Res Commun* 2005;333:1348–52. [PubMed: 15979582]
20. Gorr TA, Cahn JD, Yamagata H, Bunn HF. Hypoxia-induced synthesis of hemoglobin in the crustacean *Daphnia magna* is hypoxia-inducible factor-dependent. *J Biol Chem* 2004;279:36038–47. [PubMed: 15169764]
21. Bichet S, Wenger RH, Camenisch G, et al. Oxygen tension modulates β -globin switching in embryoid bodies. *FASEB J* 1999;13:285–95. [PubMed: 9973316]
22. Dewever J, Frérart F, Bouzin C, et al. Caveolin-1 is critical for the maturation of tumor blood vessels through the regulation of both endothelial tube formation and mural cell recruitment. *Am J Pathol* 2007;171:1619–28. [PubMed: 17916598]
23. Lin M, Yu J, Murata T, Sessa WC. Caveolin-1-deficient mice have increased tumor microvascular permeability, angiogenesis and growth. *Cancer Res* 2007;67:2849–56. [PubMed: 17363608]
24. Faivre-Fiorina B, Caron A, Fassot C, et al. Presence of hemoglobin inside aortic endothelial cells after cell-free hemoglobin administration in guinea pigs. *Am J Physiol* 1999;276:H766–70. [PubMed: 9950880]
25. Moestrup SK, Møller HJ. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med* 2004;36:347–54. [PubMed: 15478309]
26. Fabrick BO, Dijkstra CD, van den Berg TK. The macrophage scavenger receptor CD163. *Immunobiology* 2005;210:153–60. [PubMed: 16164022]
27. Kruckeberg WC, Doorenbos DI, Brown PO. Genetic differences in hemoglobin influence on erythrocyte oxidative stress hemolysis. *Blood* 1987;70:909–14. [PubMed: 3651609]
28. Poole RK, Hughes MN. New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol Microbiol* 2000;36:775–83. [PubMed: 10844666]

29. Gladwin MT, Ognibene FP, Pannell LK, et al. Relative role of heme nitrosylation and beta-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. *Proc Natl Acad Sci USA* 2000;97:9943–8. [PubMed: 10954746]
30. Roesch-Ely M, Nees M, Karsai S, et al. Proteomic analysis reveals successive aberrations in protein expression from healthy mucosa to invasive head and neck cancer. *Oncogene* 2007;26:54–64. [PubMed: 16819514]
31. Ramage JM, Metheringham R, Conn A, et al. Identification of an HLA-A*0201 cytotoxic T lymphocyte epitope specific to the endothelial antigen Tie-2. *Int J Cancer* 2004;110:245–50. [PubMed: 15069689]
32. Dong Y, Qian J, Ibrahim R, Berzofsky JA, Khleif SN. Identification of H-2D^b-specific CD8+ T-cell epitopes from mouse VEGFR2 that can inhibit angiogenesis and tumor growth. *J Immunother* 2006;29:32–40. [PubMed: 16365598]
33. Wada S, Tsunoda T, Baba T, et al. Rationale for antiangiogenic cancer therapy with vaccination using epitope peptides derived from human vascular endothelial growth factor receptor 2. *Cancer Res* 2005;65:4939–46. [PubMed: 15930316]
34. Ishizaki H, Tsunoda T, Wada S, Yamauchi M, Shibuya M, Tahara H. Inhibition of tumor growth with antiangiogenic cancer vaccine using epitope peptides derived from human vascular endothelial growth factor receptor 1. *Clin Cancer Res* 2006;12:5841–9. [PubMed: 17020992]
35. Lu C, Kamat AA, Lin YG, et al. Dual targeting of endothelial cells and pericytes in antivascular therapy for ovarian carcinoma. *Clin Cancer Res* 2007;13:4208–17.
36. Evel-Kabler K, Song XT, Aldrich M, Huang XF, Chen SY. SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. *J Clin Invest* 2006;116:90–100. [PubMed: 16357940]
37. Belladonna ML, Grohmann U, Bianchi R, Ayroldi E, Surace D, Puccetti P, Fioretti MC. The role of IL-12 in the induction of an immune response to a tumor/self peptide: prevention and reversion of anergy. *J Chemother* 1998;10:157–9. [PubMed: 9603645]

Figure 1. Intratumoral delivery of DC.IL12 is therapeutically effective against s.c. CMS4 tumors and promotes poly-specific CD8⁺ T cell-mediated responses *in vivo*

In panel **A.**, day 5 DCs were left untreated (control) or infected with rAd.ψ5 or rAd.IL-12 at MOI = 50. After 48h, culture supernatants were analyzed using an IL-12p70 ELISA. Results are representative of three independent experiments performed. In panel **B.**, control or gene-modified DC (10^6) were injected into established s.c. CMS4 tumors on days 7 and 14 post-tumor inoculation and tumor growth monitored through day 28. All cohorts contained 5 mice/group, with results representative of 2 independent experiments performed. On day 28, CMS4 tumors were resected from untreated mice, digested into single-cell suspensions and peptides extracted by mild acid elution. De-salted peptides were then resolved into 50 fractions using RP-HPLC and a 10-100% acetonitrile gradient, with protein content monitored at 214 nm (panel **C.**). In panel **D.**, after removing organic solvents by lyophilization and reconstitution with PBS, individual fractions were loaded onto syngenic control DC and assessed for their ability to elicit IFN-γ production (monitored by ELISA) from CD8⁺ splenic T cells isolated on d28 from mice treated in panel **B.** above. Results depicted are representative of 2 independent experiments performed.

Figure 2. MS analyses of dominant peptide species in bioactive HPLC fractions

HPLC fractions 19/20 identified in Fig. 1D as exhibiting the greatest degree of recognition by protective anti-tumor CD8⁺ T cells were submitted for mass spectrometric analyses (MALDI-TOF). Fraction 19 contained a predominant doubly-charged peptide (m/z 606) that yielded a Mr = 1211.4 as a singly-charged species (panel A.). MS/MS fragmentation of this doubly-charged species was then performed by microcapillary LC-ESI-tandem MS analysis. This revealed a deduced sequence of VVYPWTQRY for the Mr = 1211.4 peptide species (panel B.). Similarly, fraction 20 contained a dominant doubly-charged peptide (m/z 663) that yielded a Mr = 1324.8 as a singly-charged species (panel C.). MS/MS analysis of the Mr = 1324.8 mass ion revealed a sequence of XVVYPWTQRY (X = Leucine or Isoleucine; both with Mr = 113; panel D.). Based on protein database searches for identity, both sequences appear to derive from either murine HBB1 and/or HBB2 proteins, each of which contain the peptide sequence LVVYPWTQRY.

Figure 3. The HBB₃₃₋₄₂ peptide is recognized by H-2L^d-restricted, splenic CD8+ T cells isolated from CMS4 tumor-bearing mice treated with DC.IL12 intratumoral administration and such T cells are enriched in the spleens of mice vaccinated with DC.IL-12/HBB peptide

CD8+ splenocytes were harvested from CMS4 tumor-bearing mice treated with i.t. DC.IL12 therapy 28 days after tumor inoculation, then re-stimulated *in vitro* for 5 days with irradiated CMS4 tumor cells as outlined in Materials and Methods. In panel **A.**, responder T cells were than co-cultured with CMS4 tumor cells or with the irrelevant H-2^d MethA sarcoma cell line (in the absence or presence of HBB₃₃₋₄₂ peptide) for 48h. In replicate wells, blocking anti-H-2K^d, -D^d or -L^d mAbs were added to determine the class I restriction elements used for specific CD8+ T cell recognition of target cells. Specific T cell responses were determined by quantitation of secreted IFN- γ by ELISA. * indicates group differences with p < 0.05. In panel **B.**, intracellular staining for IFN- γ was performed on control vs. peptide-stimulated T cells isolated from the spleens of mice vaccinated with DC.IL12 alone or DC.IL12 pulsed with the HBB₃₃₋₄₂ peptide. T cells were co-stained using anti-CD8 mAb and analyzed by flow cytometry, as outlined in Materials and Methods. Data are representative of 2 independent experiments performed.

Figure 4. Mice vaccinated with the HBB₃₃₋₄₂ peptide reject a subsequent challenge with HBB^{neg} tumor cell lines

In panel **A.**, BALB/c mice (5 mice/group) were either not vaccinated or vaccinated with 10^6 DC.IL12 alone (▼), 10^6 DC.IL12 cells pulsed with the HBB₃₃₋₄₂ peptide (●) or 10^6 DC.ψ5 cells pulsed with the HBB peptide (○) on days -14 and -7. On day 0, mice were challenged with the CMS4 (5×10^5), MethA (2.5×10^6), TS/A (10^5) or CT26 (10^5) tumor cell lines and monitored for up to 4 weeks. Data are representative of 2 independent experiments performed in each case. Tumor growth in naïve, non-vaccinated animals was indistinguishable from that observed for mice vaccinated with DC.IL12 alone (data not shown). In panel **B.**, the experimental protocol in panel **A.** above was repeated with the exception that depleting anti-CD4 or anti-CD8 mAbs were injected i.p. into mice on d-3, -2 and -1 relative to CMS4 tumor challenge. Tumor size for each cohort was then compared vs. untreated control mice on day 14. *indicates significant group differences, $p < 0.05$. In panel **C.**, to determine the HBB (vs. control β-actin) mRNA expression status of the CMS4, MethA, TS/A and CT26 tumor cell lines, RT-PCR was performed and compared to peripheral blood mononuclear cells (PBMC) obtained from normal mice via tail venipuncture. -cDNA indicates control RT-PCR performed in the absence of template mRNA.

Figure 5. Regressing CMS4 tumors in HBB peptide vaccinated mice exhibit elevated CD8+ T cell infiltration and reduced expression of CD31 VEC, SMA+ pericytes and HBB protein *in situ*
 The experiment outlined in Fig. 4A was repeated and CMS4 tumor lesions surgically resected from mice vaccinated with DC.IL12/no peptide vs. DC.IL12/HBB peptide on day 14 post-tumor challenge. In panel **A.**, after sectioning, tissues were evaluated for CD8+ T cells (sub-panels a, b), CD4+ T cells (sub-panels c, d), CD31+ VEC (sub-panels e-h), SMA+ pericytes (sub-panels e-f) and HBB (sub-panels g, h) using multi-color confocal immunofluorescence. Data obtained from unvaccinated mice was comparable to that derived from control DC.IL12/no peptide vaccinated mice (data not shown). In panel **B.**, the number of CD31+ vessels per high-power field (HPF, panel **B.**) and the percentage of CD31+ vessels with associated SMA+ pericytes (panel **C.**) was quantitated for subpanels e. vs. f.; * indicates $p < 0.05$.

Figure 6. Pericytes are the major cell population within the progressor tumor microenvironment expressing HBB

In panel **A.**, CMS4 tumors growing progressively in untreated BALB/c mice were harvested on day 28, sectioned and co-stained for the pericyte markers NG2 or SMA vs. HBB, then analyzed by 2-color confocal immunofluorescence. In panel **B.**, the percentage of CD31+ (from Fig. 5Ag) or NG2+ or SMA+ cells (from Fig. 5Ag and Fig. 6A) co-expressing HBB was quantitated, with significant group differences indicated with an asterisk (*; $p < 0.05$). In panel **C.**, the CMS4 tumor-associated vasculature was analyzed for coordinate expression of HBB vs. CD163 (a hemoglobin scavenger receptor) using immunofluorescence microscopy, as outlined in Materials and Methods. Data are representative of 3 independent experiments performed.