Published in final edited form as:

Cancer Res. 2010 July 15; 70(14): 5778–5787. doi:10.1158/0008-5472.CAN-09-2538.

DC-HIL/Gpnmb promotes growth of melanoma in mice by inhibiting the activation of tumor-reactive T cells

Mizuki Tomihari^{1,2}, Jin-Sung Chung¹, Hideo Akiyoshi^{1,3}, Ponciano D. Cruz Jr.¹, and Kiyoshi Ariizumi¹

¹ Department of Dermatology, The University of Texas Southwestern Medical Center and Dermatology Section (Medical Service), Dallas Veterans Affairs Medical Center, Dallas, TX

² Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan

³ Laboratory of Advanced Diagnosis and Treatment, Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan

Abstract

DC-HIL/Gpnmb expressed on antigen-presenting cells (APC) attenuates T cell activation by binding to syndecan-4 (SD-4) on activated T cells. Since DC-HIL/Gpnmb is expressed abundantly by mouse and human melanoma lines, we posited that melanoma-associated DC-HIL/Gpnmb exerts similar inhibitory function on melanoma-reactive T cells. We generated siRNA-transfected B16F10 melanoma cells to completely knock-down DC-HIL/Gpnmb expression, with no alteration in cell morphology, melanin synthesis, nor MHC class I expression. This knockdown had no effect on B16F10 proliferation *in vitro* nor entry into the cell cycle following growth stimulation, but it markedly reduced growth of these cells *in vivo* following their subcutaneous injection into syngeneic immunocompetent (but not immunodeficient) mice. This reduction in tumor growth was due most likely to an augmented capacity of DC-HIL knocked-down B16F10 cells (compared to controls) to activate melanoma-reactive T cells as documented *in vitro* and in mice. Whereas DC-HIL knock-down had no effect on susceptibility of melanoma to killing by cytotoxic T cells, blocking SD-4 function enhanced reactivity of CD8⁺ T cells to melanoma-associated antigens on parental B16F10 cells. Using an assay examining spread to lung following intravenous injection, DC-HIL knocked-down cells produced lung foci at similar numbers compared to that produced by control cells, but the size of the former foci was significantly smaller than the latter. We conclude that DC-HIL/Gpnmb confers upon melanoma the ability to downregulate activation of melanoma-reactive T cells, thereby allowing melanoma to evade immunologic recognition and destruction. As such, the DC-HIL/SD-4 pathway is a potentially useful target for anti-melanoma immunotherapy.

Keywords

Co-inhibitory molecule; DC-HIL; immune evasion; melanoma; T cells

Address Correspondence to: Dr. Kiyoshi Ariizumi, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-9069, kiyoshi.ariizumi@utsouthwestern.edu.

Note: M. Tomihari and J-S. Chung contributed equally to this work.

P.D. Cruz and K. Ariizumi contributed equally to this work.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Introduction

The success or failure of immune responses mounted against tumors is determined by the net result of competing mechanisms defending against or permitting tumor growth and metastasis. Host defense components include natural killer cells and cytotoxic T lymphocytes (CTL) that can lyse the tumor (1). On the other hand, tumors can employ a variety of mechanisms that allow their escape from immunologic surveillance and destruction, including poorly expressed tumor-associated antigens (TAA), self-tolerance to TAA, down-regulated major histocompatibility complex (MHC) molecules, secretion of immunosuppressive cytokines (TGF, IL-10, and VEGF), and deficient T cell co-stimulation (2;3).

T cell activation results from signals delivered by antigen presenting cells (APC) to the antigen (Ag)-specific T cell receptor (TCR) and to co-stimulatory or co-inhibitory receptors on T cells. The principal co-stimulatory signal is transmitted by CD80 or CD86 on APC to the CD28 receptor on T cells, which amplifies TCR-activation signals (4). By contrast, co-inhibitory molecules inhibiting TCR signals include: programmed cell death-1 (PD-1) and its ligands PD-L1 (B7-H1) and PD-L2 (5–7); B- and T-lymphocyte attenuator (BTLA) and herpesvirus entry mediator (HVEM) (4;8); and Tim-3 ligand/Galectin-9 (on CD4⁺ T cells) and Tim-3 (on APC and Th1 effector cells) (9–11). Like APC, tumors also may take advantage of co-inhibitory ligands that compromise immunosurveillance by suppressing activation of tumor-reactive CTL, reducing susceptibility of tumors to CTL-lysis, and/or inducing their apoptosis (12;13).

Subtractive cDNA cloning of dendritic antigen-presenting cells (DC) led us to discover DC-HIL, a highly-glycosylated (95–120 KDa) type I transmembrane receptor expressed constitutively at high levels by many APC subsets and at lower levels by some nonlymphoid cells (14). DC-HIL was also identified by others, and given a variety of names including: glycoprotein nmb (Gpnmb) (15), osteoactivin (16), and hematopoietic growth factor-inducible neurokinin-1 type (HGFIN) (17). The extracellular domain of DC-HIL contains a cell-adhesion (RGD) motif, a proline-rich region, and an Ig-like polycystic kidney disease (PKD) domain shared by PKD-susceptible gene products (18). Recently, we found DC-HIL to inhibit T cell activation by binding syndecan-4 (SD-4) on activated (but not resting) T cells in mice and humans (19–21). This binding attenuates strongly the T cell response to anti-CD3 Ab, thereby blocking T cell production of pro-inflammatory cytokines and T cell entry into the cell cycle. In mice, we showed the DC-HIL/SD-4 pathway to inhibit T cell-mediated contact hypersensitivity (19).

Unlike other known co-inhibitors of T cell activation, DC-HIL/Gpnmb is expressed constitutively and abundantly by mouse melanoma lines, a finding that prompted us to question whether DC-HIL/Gpnmb on melanoma provides resistance to immunologic recognition and destruction by melanoma-reactive T cells. Our results indicate that DC-HIL/Gpnmb promotes growth of B16F10 melanoma in syngeneic mice by inhibiting activation of tumor-reactive T cells, thereby making the DC-HIL/SD-4 pathway a potentially useful target for anti-melanoma immunotherapy.

Materials and Methods

Mice and cell culture

Female C57BL/6 mice (5–8 weeks old) were purchased from Harlan Breeders (Indianapolis, IN); nu/nu and pmel-1 TCR transgenic mice (B6.Cg-*Thy1^a/Cy* Tg(Tcr α Tcr β)8Rest/J) from Jackson Laboratory (Bar Harbor, Maine); and C57BL6-*Tg(OT-1)-RAG1^{tm1Mom}* from Taconic (Hudson, NY). Following National Institutes of Health guidelines, these animals

were housed and cared for in the pathogen-free facility of the Institutional Animal Care Use Center of The University of Texas Southwestern Medical Center. All animal protocols were approved by the Center. B16F10 melanoma and EL-4 T lymphoma were purchased from American Type Culture Collection (ATCC, Manassas, VA), and maintained in Dulbecco's MEM (DMEM) supplemented with 10% FCS.

RT-PCR Analysis

Total RNA (1 µg) isolated from B16F10 cells or dendritic cells (DC) was converted to cDNA by reverse transcriptase (Life Technologies, Inc., Rockville, MD) (22). An aliquot (typically 5%) was used for PCR amplification (22) using the primers: for DC-HIL, 5'-CTGCCAAGCGATTTTCGTGATGTG-3' and 5'-CCTGTCCGGGAACCTGAGATGC-3'; for PD-L1, 5'-CCCATACCGCAAAATCAACCAG-3' and 5'-CAACGCCACATTTCTCCACATCTA-3'; for PD-L2, 5'-CACCGTGACAGCCCCTAAAGAAGT-3' and 5'-GGGGTCCTGATGTGGCTGGTGT-3'; for HVEM, 5'-CATCTCTGCCCAGCCCTCAT-3' and 5'-GCTATCCCAACTCCCACTATCACA-3'; for Tim-3, 5'-TGCCAAGTACTCATGTTTTTCAGGT-3' and 5'-CTCTCCGTGGTTAGGGTTCTTG-3'; or for β-actin, 5'-GAGCGGGAAATCGTGCCTGACATT-3' and 5'-GAAGGTAGTTTCGTGGATGCC-3'. Following 30-cycles of amplification, PCR products were separated electrophoretically on 1% agarose gel.

Plasmid vectors

We constructed a siRNA-expressing lentivector consisting of DC-HIL-targeted short hairpin (sh) RNA (58 base oligonucleotides, the sense strand, 5'-GATCCG-GGAAGTTGTCTGATGAGATCT-CGAA-AGATCTCATCAGACAAGTTCC-TTTTTG-3'; and the antisense strand, 5'-AATTCAAAAA-GGAAGTTGTCTGATGAGATCT-TTCG-AGATCTCATCAGACAAGTTCC-CG-3', in which the italic nucleotides are *Bam*HI site in the sense strand and *Eco*RI in the antisense strand synthesized by Integrated DNA Technologies (Coralville, IA) and ligated to the downstream of H1 RNA polymerase III promoter in pSIF1-H1-copGFP shRNA lentivector (System Biosciences, Mountain View, CA) that contains a CMV-driven-fluorescent copepod GFP (copGFP). pSIF1-H1-copGFP without a shRNA insert served as control lentivector. Packaging of pSIF constructs in pseudoviral particles and their titration were performed according to established protocols (23).

Generation of stable transfectants

To generate B16F10 cells knocked-down for DC-HIL, B16F10 cells (1×10^4) were infected with control or DC-HIL-shRNA lentivector at a multiplication of infection (MOI) of 20. Next day, GFP⁺ cells were enriched repeatedly by FACS sorting until more than 90% of cells were GFP⁺. To minimize alterations due to *in vitro* expansion, an original batch of positive cells was divided into several aliquots and stored in liquid nitrogen until needed.

Western blot analysis

Whole cell extracts were prepared from B16F10 cells by lysis with 0.3% Triton X-100/DPBS for 15 min, followed by centrifugation for 20 min at $10,000 \times g$ (24). An aliquot (40 µg) of extract was applied to SDS/4-15% gradient PAGE, followed by immunoblotting using UTX-103 rabbit anti-DC-HIL mAb (25) (2 µg/ml) or anti-β-actin Ab (Abcam, Cambridge, MA). For examining expression of DC-HIL and gp100 in exosomes, an aliquot (20 µg) was applied to immunoblotting using UTX-103 or 1 µg/ml mouse anti-human melanosome (gp100) mAb (clone HMB45, DakoCytomation, Inc., Carpinteria, CA). Color was developed by HRP-secondary Ab (1:10,000 dilution, Jackson ImmunoResearch

Laboratories Inc., West Grove, PA) for 1 h and ECL plus system (Amersham Pharmacia Biotech, Piscataway, NY).

Flow cytometry

To analyze surface expression, B16F10 cells (1×10^5) were incubated with UTX-103 mAb, biotinylated anti-H-2D^b (eBioscience, San Diego, CA), or isotypic control Ab (each 5 µg/ml). To measure expression of H-2K^b molecule bound to ovalbumin (OVA) peptide, B16 F10 cells were preincubated with 2 µg/ml OVA₂₅₇₋₂₆₄ (SIINFEKL) for 2 h prior to staining with 5 µg/ml PE-conjugated anti-H-2K^b (K^b-SIINFEKL, eBioscience). After extensive washing, cells were labeled fluorescently with secondary Ab (5µg/ml PE- or FITC-anti-rabbit IgG, Jackson ImmunoResearch Laboratories Inc., or 5µg/ml PE-streptavidin, BD Biosciences, San Jose, CA). Fluorescence intensity of stained cells was analyzed by FACS Calibur (BD Biosciences).

MTT assay

Tumor cell growth *in vitro* was measured by the colorimetric MTT assay. Briefly, exponentially growing control or knockdown transfectants were washed thoroughly and incubated for 24 h in serum-free media to synchronize cell growth. Thereafter, 1×10^4 cells/well were seeded in 96-well plates (in triplicate) and cultured in complete DMEM. Cell growth was determined each day by adding 10µl MTT Reagent (from ATCC). Cells were then incubated at 37°C for 2 h, and 100 µl Detergent Reagent (ATCC) added and left at room temperature in the dark for 2 h. Absorbance was determined at 595 nm.

Cell cycle analysis

B16F10 melanoma cells were seeded on a Petri dish (5×10^5 cells/dish) and starved for 24h in serum-free media to synchronize. Proliferation was stimulated by adding 10% FCS-media and culturing for 18 h. These cells were subjected to cell cycle analysis using BrdU labeling and the BrdU flow kit (BD Biosciences), following manufacturer's recommendations. BrdU incorporation was assessed by staining with biotinylated anti-BrdU Ab (BioLegend, San Diego, CA) and APC-conjugated streptavidin (eBioscience). BrdU labeling and DNA content (stained by 7-AAD) on a per-cell basis were analyzed by flowcytometry.

In vivo tumor growth

C57BL/6 or nu/nu mice were inoculated with control or knockdown B16F10 transfectants by subcutaneous injection of 2×10^5 cells in 50 µl of sterile DPBS into the right shaved flank. Tumor growth was monitored daily by measuring tumor perpendicular diameters with a metric caliper, and tumor volume was estimated as ($d^2 \times D \times 0.5$), where d and D are the minor and major diameters, respectively (26). For ethical reasons, animals were sacrificed when tumors grew to a volume greater than 5 cm³.

Exosome preparation

Culture supernatant was harvested from 72 h culture of confluent melanoma cells (C-B16 and Kd-B16). The supernatant was centrifuged at $5,000 \times g$ for 30 min at 4°C to remove cell debris and the obtained supernatant was then ultracentrifuged at $100,000 \times g$ for 6 h at 4°C (27). The pellet (membrane fraction) was recovered and measured for protein concentration and resuspended in DPBS.

Ex vivo analyses of T cells

To examine the capacity of B16F10 transfectants to stimulate T cells, CD8⁺ T cells isolated from OT-I transgenic mice, using the CD8⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA), were seeded at a density of 2×10^5 cells/well in 96-microplates (in triplicate) and

cocultured with B16F10 transfectants (2×10^4 cells/well) pulsed with 2 $\mu\text{g/ml}$ OVA₂₅₇₋₂₆₄ for 3 h, then subsequently treated with mitomycin C (MMC, 25 ng/ml) for 1 h. After culturing for 2 d, IL-2 and/or IFN- γ production was measured using an ELISA kit (eBioscience). In some experiments, instead of naive OVA-specific T cells, TAA-immunized CD8⁺ T cells were used: C57BL/6 mice were immunized by footpad injection of 30 μl TiterMax adjuvant (Sigma-Aldrich) containing TAA peptide mixture (hgp100 aa25-33 and TRP-2 aa180-188, each 20 μg , both from ANASPEC, San Jose, CA). 17 d-post-immunization, cells were prepared from spleen and lymph nodes (LN) of the mice, which were then stimulated *in vitro* with γ -irradiated (3000 Gy) syngeneic spleen cells that were pulsed with the peptide mixture (2 $\mu\text{g/ml}$ hgp100 and 2 $\mu\text{g/ml}$ TRP-2). After 10 d in culture, CD8⁺ T cells were isolated and similarly cocultured with MMC-treated B16F10 transfectants for 2 d. OVA-immunized CD8⁺ T cells also were prepared similarly. To measure IFN- γ -producing cells, enzyme-linked immunospot (ELISPOT) method was used: B16F10 transfectants (each 2×10^5 cells) were *s.c.* injected into C57BL/6 mice. 14 d-post-inoculation, draining LN cells were prepared and cocultured with γ -irradiated syngeneic spleen cells pulsed with the TAA peptide mixture (each 2 $\mu\text{g/ml}$) for 7 d. Thereafter, cells were extensively washed, adjusted to 2×10^6 cells/ml, 2-fold serially diluted, and seeded on 96-well PVDF membrane ELISPOT plate (MultiScreen_{HTS}-IP Filter plate, Millipore, Billerica, MA). The number of IFN- γ -producing cells was counted and calculated per 2×10^6 LN cells. To examine an inhibitory effect of exosomes on T cell activation, CD8⁺ T cells (2×10^5 /well) were isolated from day 6 culture of spleen cells from pmel-1 TCR transgenic mice with 1 $\mu\text{g/ml}$ hgp100 peptide (EGSRNQDWL, from ANASPEC) and cocultured with MMC-treated B16F10 cells (2×10^4 /well) in the presence of indicated doses of exosomes for 2 d. IL-2 and IFN- γ production was measured.

Cytotoxicity assay

B16F10 or EL-4 tumor cells (1×10^6) were preincubated with or without 2 $\mu\text{g/ml}$ OVA₂₅₇₋₂₆₄ or TAA peptide mixture (each 2 $\mu\text{g/ml}$) for 3 h, and then labeled with 100 μCi of Na⁵¹Cr for 1 h. OVA-specific or TAA-immunized T cells were added to the labeled tumor cells (1×10^4 /well) at varying effector-target (ET) ratios, and incubated for 4 h. Specific ⁵¹Cr release was determined as described previously (28).

Experimental lung metastasis

B16F10 transfectant cells (2×10^5) were harvested and suspended in 200 μl of DPBS and injected in the lateral tail vein of C57BL/6 mice. Lungs were harvested 11 or 14 d post-injection, and their total weight, number of metastatic foci, and melanin content examined (29). Melanin was quantified in lungs as described previously (30).

Statistical analysis

Groups were compared using Student's *t* test for both *in vitro* and animal experiments.

Results

B16F10 melanoma cells express DC-HIL mRNA constitutively at a level higher than other known co-inhibitory ligands

To determine the expression profile of co-inhibitory ligand genes by melanoma cells, we used RT-PCR to analyze mRNA expression of DC-HIL, PD-L1, PD-L2, HVEM, and Tim-3. Bone marrow-derived dendritic cells (BM-DC) served as positive control since these cells express all of the aforementioned ligands. DC-HIL mRNA was expressed constitutively by B16F10 melanoma cells at a level as high as in BM-DC (Fig. 1A); PD-L1 was present but at a much lower level, and the other co-inhibitory ligands not at all. IFN- γ treatment did not

change mRNA expression level of DC-HIL, whereas it upregulated mRNA expression of PD-L1 markedly by a level close to that of DC-HIL.

Knocked-down DC-HIL expression did not change melanoma phenotype and proliferation *in vitro*

To study the function of DC-HIL in melanoma, we used siRNA to generate B16F10 cells knocked-down for DC-HIL expression. We synthesized 5 different shRNA oligonucleotides and inserted each into a lentivector. The efficacy of each shRNA to knock down DC-HIL expression was assessed by reduction in protein expression. The shRNA showing highest efficacy (data not shown) was used to generate the knocked-down transfectant. To avoid clonal effects, infected B16F10 cells were enriched by repeated FACS sorting instead of cloning by limiting dilution. One batch of infected B16F10 cells was assayed by Western blotting for DC-HIL protein (Fig. 1B). Compared to control B16F10 cells (referred to as C-B16) infected with control lentivector with no shRNA sequence, DC-HIL-shRNA-infected cells (Kd-B16) showed almost no DC-HIL expression (β -actin expression was unchanged). Consistent with the very low level of total protein expression, surface expression of DC-HIL was hardly detected even in the Kd-B16 cells treated with IFN- γ (Supplementary figure S1). There were no morphologic changes in Kd-B16 cells, and no significant differences in expression of MHC class I molecules H-2D^b and OVA-associated H-2K^b between the two B16F10 transfectants (Supplementary Fig. S2). Melanin content of Kd-B16 cells was almost identical to that of C-B16 cells (Supplementary Fig. S3). Finally, there was also no difference in the MTT proliferation assay between the two transfectants (Fig. 1C). To more precisely analyze growth capacity, the two B16F10 transfectants were analyzed for entry into the cell cycle in response to growth stimulation. Cells were synchronized by starvation and stimulated to grow by adding FCS-containing media for 18 h; cell cycle analysis was performed using BrdU and 7-AAD (Fig. 1D). Kd-B16 and C-B16 cells were sorted into each cell cycle phase at close to equal frequencies. Our results indicate that knocking down DC-HIL expression produces no significant change in morphology, melanin synthesis, and MHC class I expression. Moreover, the knockdown had no effect on growth *in vitro* nor entry into cell cycle following growth stimulation.

DC-HIL-knocked-down B16F10 cells show reduced growth in wild-type (immunocompetent), but not immunodeficient mice

We evaluated tumor growth of the transfectants in syngeneic C57BL/6 mice (Fig. 2A). C-B16 or Kd-B16 melanoma cells were inoculated *s.c.* into the right flank of mice, and tumor volume measured daily. Kd-B16 cells grew significantly slower than control cells (tumor volume of $5.08 \pm 0.98 \text{ cm}^3$ for C-B16 vs. 1.61 ± 0.68 for Kd-B16 at 24 days after inoculation, $p=2 \times 10^{-8}$). Mice bearing C-B16 tumor started to die on day 16, and all of these animals died by day 26. By contrast, Kd-B16 tumor-inoculated mice started dying on day 27, with some survivors through day 43 (Fig. 2B). Most importantly, Kd-B16 tumor growth in immunodeficient nu/nu mice was not reduced compared to controls (Fig. 2C). We also questioned whether concomitant inoculation of C-B16 and Kd-B16 cells (mixed in equal numbers) suppresses growth of subcutaneously injected C-B16 tumor (Fig. 2D). Indeed, growth of the mixed cells was reduced significantly throughout the experiment, compared to C-B16 cells alone, but not as much as Kd-B16 alone (Fig. 2D1). Consistent with this growth reduction, the survival rate of mice injected with the mixed cells was much higher compared to mice treated with C-B16 alone (Fig. 2D2). These results indicate that reduced tumor growth produced by knocking-down DC-HIL requires host immune function and that Kd-B16 cells have an adjuvant effect.

To further validate our results, we examined the effect of nonspecific siRNA on tumor growth. B16F10 cells transfected with nonspecific sequence (NS) siRNA (NS-B16)

targeting the firefly luciferase gene showed no change in DC-HIL protein expression (Supplementary figure S4B). Growth of NS-B16 tumor was similar to C-B16 by day 20, and thereafter slightly slower than C-B16 (Supplementary figure S4C). We then compared effects of siRNA targeting DC-HIL vs. PD-L1 gene on tumor growth. B16F10 cells transfected with PD-L1-siRNA showed markedly reduced PD-L1 expression in whole cell extracts and on the cell surface, but β -actin and DC-HIL levels were unchanged (Supplementary figure S4A and S4B). PD-L1-knocked down B16F10 tumor showed growth and survival rates very similar to NS-B16 and C-B16, whereas, in the same setting, Kd-B16 tumor had markedly reduced growth and increased survival (Supplementary figure S4C and D). These results indicate that control siRNA sequences had very little to no effect on tumor growth of B16F10 cells and that knocking down DC-HIL is more effective than knocking down PD-L1 in reducing tumor growth.

DC-HIL-knockdown heightens activation of melanoma-reactive T cells, but not susceptibility to CTL killing

Having found DC-HIL on APC to inhibit T cell activation after binding to SD-4 on T cells, we hypothesized that melanoma-associated DC-HIL exerts similar function. We thus examined the impact of knocking down DC-HIL on the immunogenicity of T cells for B16F10 melanoma cells. Because B16F10 melanoma is inherently weak immunogenically, we employed OVA as a surrogate tumor-associated Ag (TAA). B16F10 transfectants were pulsed with MHC class I-associated OVA peptide (OVA₂₅₇₋₂₆₄) and then allowed to stimulate CD8⁺ T cells isolated from unprimed OT-I transgenic mice. T cell activation was measured by secretion of IL-2 and IFN- γ (Fig. 3A). Kd-B16 cells stimulated T cells to produce IL-2 and IFN- γ at levels 50% higher than C-B16 cells.

We next questioned whether SD-4 is involved in the enhanced T cell-stimulatory capacity. OVA-specific CD8⁺ CTL were generated by immunizing OT-I mice with OVA peptide followed by *in vitro* reactivation, and finally examined by flow cytometry for SD-4 expression (Fig. 3B). All CTL expressed the CD69 activation marker, and half of CD69⁺ CTL co-expressed SD-4. To examine involvement of SD-4 in T cell immunogenicity by melanoma cells, we assayed activation of CD8⁺ T cells by parental B16F10 cells in the presence of soluble anti-SD-4 mAb (to block endogenous SD-4 function on CTL) or control IgG (Fig. 3C). OVA-pulsed parental melanoma cells stimulated CD8⁺ T cells to secrete IL-2 at a level similar to that in C-B16 cells. This IL-2 secretion was enhanced by addition of anti-SD-4 mAb (but not by control IgG) in a dose-dependent manner.

We next assessed susceptibility of B16F10 transfectants to lysis by CTL (Fig. 3D). OVA-specific CD8⁺ CTL lysed OVA-pulsed C-B16 very efficiently (50% target lysis), but not unpulsed B16F10 cells. These CTL also were capable of lysing OVA-pulsed Kd-B16 cells, albeit at a lower efficiency (insignificant difference, $p < 0.18$). Thus, both control and DC-HIL knocked-down B16F10 cells appear to be similarly sensitive to CTL cytotoxicity.

To ascertain the effect of knocking down DC-HIL, we used hgp100 and TRP-2 Ag that have been used as TAA in anti-melanoma immunotherapy (31;32). CD8⁺ CTL were prepared by *in vitro* restimulation of lymph node (LN) cells isolated from C57BL/6 mice immunized with the two peptides, and finally cocultured with C-B16 or Kd-B16 cells (Fig. 4). Consistent with results of studies using OVA peptide, CTL were more strongly activated by Kd-B16 cells than by control cells (Fig. 4A). Again, there was no significant difference in susceptibility to cell lysis by hgp100/TRP-2-specific CTL (Fig. 4B). Using these TAA mouse models, we assayed IFN- γ -producing cells from LN of mice challenged with B16F10 tumors (Fig. 4C). C57BL/6 mice were tumor-challenged by *s.c.* injection of B16F10 cells, 2 weeks after which LN cells were prepared and restimulated *in vitro* with spleen cells pulsed with hgp100 and TRP-2 peptides. IFN- γ -secreting cells were counted by ELISPOT assay.

Mice challenged with Kd-B16 cells produced a significantly higher number of IFN- γ -secreting LN cells than control cells. Altogether, these results indicate that knocking-down DC-HIL heightens the capacity of B16F10 cells to activate tumor-reactive T cells, but does not significantly alter susceptibility to CTL lysis.

B16F10-released exosomes contain DC-HIL that inhibits T cell activation triggered by melanoma Ag

Since melanoma cells release exosomes (extracellular melanosomes or microvesicles) that promote immunosuppression (33), we examined the possibility that DC-HIL is also packed into exosomes that can inhibit T cell activation (Fig. 5). Exosomes prepared from C-B16 cells contained DC-HIL protein at a high level, and those of Kd-B16 cells also contained it but at an extremely low level, consistent with expression in the whole cell extracts (Fig. 1B). By contrast, both exosome preparations expressed similar levels of the melanosomal protein gp100, indicating that similar amounts of exosomes were examined. We then questioned whether these exosomes inhibit activation of CD8⁺ T cells (expressing SD-4) by melanoma (Fig. 5B). SD-4⁺CD8⁺ T cells were prepared by *in vitro* culturing spleen cells from pmel-1 TCR transgenic mice in which all CD8⁺ T cells express transgenic TCR that recognizes pmel-17 (mouse homologue of human gp100) in the context of the H-2D^b (34). These cells expressed SD-4 at a level as high as OT-I CD8⁺ T cells (data not shown). B16F10 parental cells were allowed to stimulate the pmel-1 CD8⁺ T cells in the presence of exosomes prepared from C-B16 or Kd-B16 cells (Fig. 5B). Addition of exosomes (200 μ g/ml) from C-B16 cells led to 80% reduction in IL-2 production by pmel-1 T cells (50% with 100 μ g/ml), whereas exosomes from Kd-B16 cells had no inhibitory effect on IL-2 production. Similar results were noted for effects on IFN- γ production (Fig. 5B).

DC-HIL-knockdown does not alter the capacity of melanoma to spread to lung, but it reduces growth of melanoma within lung foci

We next examined the effect of DC-HIL-knockdown on dissemination of B16F10 melanoma to lung following intravenous injection into syngeneic hosts (Fig. 6). Two weeks after infusing B16F10 transfectants into C57BL/6 mice via tail vein, mice were sacrificed and lungs assayed for weight, number of foci, and melanin content (Exp. 1). Comparing lungs of mice injected with C-B16 cells to those injected with Kd-B16 cells, the latter were lighter (0.21 ± 0.02 g vs. 0.20 ± 0.02 g, $p=0.23$) and contained more lung foci (2091 ± 240 vs. 2333 ± 261 , $p=0.12$), although these differences were not statistically significant. However, lung foci for Kd-B16-injected mice were smaller. Because the two B16F10 transfectants contain almost identical melanin content (Supplementary Fig. S2), we also evaluated melanin content, and found that for Kd-B16 cells total melanin content per lung was lower (846 ± 293 μ g vs. 611 ± 112 μ g, $p=0.11$) and melanin per focus was significantly less (0.40 ± 0.11 vs. 0.27 ± 0.05 , $p=0.03$). A second experiment (Exp. 2) reproduced the latter finding (0.68 ± 0.12 vs. 0.43 ± 0.21 , $p=0.04$). These results indicate that the impact of knocking down DC-HIL in B16F10 cells is manifest, not in the capacity for establishment in lung following intravenous injection, but in their potential for growth within lung foci.

Discussion

To determine whether melanoma-associated DC-HIL inhibits activation of tumor-reactive T cells, we knocked-down DC-HIL on B16F10 mouse melanoma cells and analyzed its effects on tumor growth. Knocking-down DC-HIL had no effect on spontaneous growth nor on proliferation following growth stimulation *in vitro*. It also had no effect on melanoma growth in immunodeficient nude mice. However, it reduced growths of flank and lung B16F10 melanomas that were established, respectively, following subcutaneous and intravenous injections into syngeneic immunocompetent mice. These tumor growth

reductions are due most likely to the inhibitory function of DC-HIL on melanoma-reactive T cells since: (1) knocking down DC-HIL in B16F10 cells enhanced these cells' capacity to stimulate melanoma-reactive T cells; (2) IFN- γ -secreting effector T cells were generated more in mice challenged with the knocked-down cells; (3) SD-4 is expressed by melanoma-reactive CTL; and (4) parental B16F10 melanoma enhanced activation of CTL when SD-4 function is blocked. By contrast, we were unable to show any significant impact of DC-HIL on susceptibility to CTL-mediated lysis or on the ability of melanoma to become established in lung following intravenous injection. Moreover, the inhibitory function of DC-HIL may be delivered to lymphoid organs distal to the melanoma site via exosomes. Altogether, our findings indicate that DC-HIL confers on melanoma the ability to inhibit activation of melanoma-reactive T cells, thereby compromising optimal anti-tumor immunity.

The Gpnmb/DC-HIL gene was identified first by Weterman et al. using subtractive cDNA cloning between highly- and lowly-metastatic human melanoma cell lines (15). To study Gpnmb function, they transfected the gene into a highly metastatic human melanoma BLM line (originally devoid of Gpnmb expression) and established transgenic BLM clones. They reported no difference in *in vitro* growth between transfectants and controls but did not show data in their paper (15). These results are consistent with ours showing no effect of DC-HIL knockdown on proliferation as assessed by MTT assay and cell cycle analysis. Two of their 3 transfectant clones showed reduced subcutaneous growth when inoculated into nude mice, and one clone exhibited reduced spontaneous metastasis in nude mice, suggesting a downregulatory role for Gpnmb in melanoma growth and metastatic potential. Differences between our and their experimental systems may account for disparate implications: (1) mouse vs. human melanoma cell lines; (2) bulk vs. cloned transfectants; (3) Gpnmb protein expression was not examined in their BLM transfectants; (4) knockdown (loss-of-function) strategy by us vs. transgenic expression (gain-of-function) by them; and (5) intravenous injection of B16F10 transfectants into syngeneic immunocompetent mice by us vs. xenografts of human melanoma into nude mice by them (thereby excluding a role for host immune responses in their studies). At a minimum, these differences render a direct comparison of outcomes difficult. At best, our demonstration that the DC-HIL/SD-4 pathway favors melanoma growth is an opportunity for investigating its potential usefulness as a target for immunotherapy.

Among co-inhibitors of T cell activation, DC-HIL most closely resembles PD-L1 in its function (19). PD-L1 is expressed by different cancers and implicated in immune evasion by tumors (35;36): Transgenic expression of PD-L1 into P815 mastocytoma (lacking PD-L1 expression) promotes its growth in syngeneic immunocompetent (but not nu/nu) mice by reducing stimulation of CTL and susceptibility to tumor lysis by CTL (13;37). PD-L1 also is expressed constitutively by B16F10 melanoma but at a much lower level than DC-HIL (37), although this expression can be upregulated markedly by treatment with IFN- γ *in vitro*, and most recently shown in a subcutaneous B16F10 tumor in mice (38). However, the role of the PD-L1 pathway in melanoma growth is not clear because of two discrepant data: In mice deficient for PD-1 (the T cell receptor for PD-L1), B16F10 melanoma cells grew at a rate similar to that in wild-type mice (37), whereas inoculation of melanoma cells into mice over-expressing PD-1 markedly enhanced their growth (38). We probed the influence of PD-L1 on B16F10 melanoma cells and found no significant effect on subcutaneous tumor growth and survival of tumor-bearing mice, consistent with previous work in PD-1-deficient mice, and supportive of DC-HIL being more important than PD-L1 in this setting.

We showed that DC-HIL is enclosed in exosomes released by B16F10 cells, which inhibit T cell activation triggered by a melanoma-associated Ag pmel-17 (mouse homolog of human gp100). Exosomes have been shown at abundant levels in body fluids of patients with melanoma (39) and they can mediate immunosuppression by promoting differentiation of

monocytes to myeloid suppressor cells (which facilitate tumor growth) (40) and by inhibiting T cell function (41). It is likely that DC-HIL is involved in exosome-mediated immunosuppression. Importantly, the inhibitory function of DC-HIL can be delivered as a form of exosome to lymphoid organs (including LN, spleen and bone marrow) distal to the melanoma site.

In summary, our findings provide a rationale basis for developing treatment modalities targeting the DC-HIL/SD-4 pathway to optimize immune surveillance of melanoma and control its growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

VA Merit Award and National Institute of Health R01 (AI064927). We thank Irene Dougherty for technical assistance and Marguerite Starr for secretary assistance.

References

1. thor SP, Becker JC, Guldberg P, Zeuthen J. In situ T cells in melanoma. *Cancer Immunol Immunother.* 1999 Oct; 48(7):386–95. [PubMed: 10501852]
2. Rivoltini L, Carrabba M, Huber V, Castelli C, Novellino L, Dalerba P, et al. Immunity to cancer: attack and escape in T lymphocyte-tumor cell interaction. *Immunol Rev.* 2002 Oct; 188:97–113. [PubMed: 12445284]
3. Chouaib S, Asselin-Paturel C, Caignard A, Blay JY. The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Immunol Today.* 1997 Oct; 18(10):493–7. [PubMed: 9357142]
4. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol.* 2002; 20:29–53. [PubMed: 11861596]
5. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med.* 1999 Dec; 5(12):1365–9. [PubMed: 10581077]
6. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med.* 2000 Oct 2; 192(7):1027–34. [PubMed: 11015443]
7. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol.* 2001 Mar; 2(3):261–8. [PubMed: 11224527]
8. Carreno BM, Collins M. BTLA: a new inhibitory receptor with a B7-like ligand. *Trends Immunol.* 2003 Oct; 24(10):524–7. [PubMed: 14552835]
9. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol.* 2005 Dec; 6(12):1245–52. [PubMed: 16286920]
10. Sabatos CA, Chakravarti S, Cha E, Schubart A, Sanchez-Fueyo A, Zheng XX, et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol.* 2003 Nov; 4(11):1102–10. [PubMed: 14556006]
11. Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, et al. Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat Immunol.* 2003 Nov; 4(11):1093–101. [PubMed: 14556005]
12. Hurwitz AA, Kwon ED, van EA. Costimulatory wars: the tumor menace. *Curr Opin Immunol.* 2000 Oct; 12(5):589–96. [PubMed: 11007364]

13. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, et al. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med.* 2002 Aug; 8(8): 793–800. [PubMed: 12091876]
14. Shikano S, Bonkobara M, Zukas PK, Ariizumi K. Molecular cloning of a dendritic cell-associated transmembrane protein, DC-HIL, that promotes RGD-dependent adhesion of endothelial cells through recognition of heparan sulfate proteoglycans. *J Biol Chem.* 2001; 276(11):8125–34. [PubMed: 11114299]
15. Weterman MA, Ajubi N, van Dinter I, Degen WG, Ruitter DJ, Bloemers HP. nmb, a novel gene, is expressed in low-metastatic human melanoma cell lines and xenografts. *Int J Cancer.* 1995; 60(1): 73–81. [PubMed: 7814155]
16. Safadi FF, Xu J, Smock SL, Rico MC, Owen TA, Popoff SN. Cloning and characterization of osteoactivin, a novel cDNA expressed in osteoblasts. *J Cell Biol.* 2001; 84(1):12–26.
17. Metz RL, Patel PS, Hameed M, Bryan M, Rameshwar P. Role of human HGFIN/nmb in breast cancer. *Breast Cancer Res.* 2007; 9(5):R58. [PubMed: 17845721]
18. Ponting CP, Hofmann K, Bork P. A latrophilin/CL-1-like GPS domain in polycystin-1. *Curr Biol.* 1999 Aug 26; 9(16):R585–R588. [PubMed: 10469603]
19. Chung JS, Sato K, Dougherty I, Cruz PD Jr, Ariizumi K. DC-HIL is a negative regulator of T lymphocyte activation. *Blood.* 2007 Feb 6; 109(10):4320–7. [PubMed: 17284525]
20. Chung JS, Dougherty I, Cruz PD Jr, Ariizumi K. Syndecan-4 mediates the coinhibitory function of DC-HIL on T cell activation. *J Immunol.* 2007 Nov 1; 179(9):5778–84. [PubMed: 17947650]
21. Chung JS, Bonkobara M, Tomihari M, Cruz PD Jr, Ariizumi K. The DC-HIL/syndecan-4 pathway inhibits human allogeneic T-cell responses. *Eur J Immunol.* 2009 Apr; 39(4):965–74. [PubMed: 19350579]
22. Ariizumi K, Meng Y, Bergstresser PR, Takashima A. IFN- γ -dependent IL-7 gene regulation in keratinocytes. *J Immunol.* 1995; 154:6031–9. [PubMed: 7751645]
23. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery *in vivo*. *Nat Biotechnol.* 1997 Sep; 15(9):871–5. [PubMed: 9306402]
24. Sato K, Shikano S, Xia G, Takao J, Chung JS, Cruz PD Jr, et al. Selective expression of vacuolar H⁺-ATPase subunit d2 by particular subsets of dendritic cells among leukocytes. *Mol Immunol.* 2006 Mar; 43(9):1443–53. [PubMed: 16144709]
25. Tomihari M, Hwang SH, Chung JS, Cruz PD Jr, Ariizumi K. Gpnmb is a melanosome-associated glycoprotein that contributes to melanocyte/keratinocyte adhesion in a RGD-dependent fashion. *Exp Dermatol.* 2009 Mar 3; 18(7):586–95. [PubMed: 19320736]
26. Rubinstein N, Alvarez M, Zwirner NW, Toscano MA, Ilarregui JM, Bravo A, et al. Targeted inhibition of galectin-1 gene expression in tumor cells results in heightened T cell-mediated rejection; A potential mechanism of tumor-immune privilege. *Cancer Cell.* 2004 Mar; 5(3):241–51. [PubMed: 15050916]
27. Araki K, Horikawa T, Chakraborty AK, Nakagawa K, Itoh H, Oka M, et al. Small Gtpase rab3A is associated with melanosomes in melanoma cells. *Pigment Cell Res.* 2000 Oct; 13(5):332–6. [PubMed: 11041209]
28. Harada M, Tamada K, Abe K, Li T, Onoe Y, Tada H, et al. Characterization of B16 melanoma-specific cytotoxic T lymphocytes. *Cancer Immunol Immunother.* 1998; 47:198–204. [PubMed: 9875672]
29. Toura I, Kawano T, Akutsu Y, Nakayama T, Ochiai T, Taniguchi M. Cutting Edge: Inhibition of Experimental Tumor Metastasis by Dendritic Cells Pulsed with α -Galactosylceramide. *J Immunol.* 1999; 163:2387–91. [PubMed: 10452972]
30. Reischer D, Heyfets A, Shimony S, Nordenberg J, Kashman Y, Flescher E. Effects of natural and novel synthetic jasmonates in experimental metastatic melanoma. *Br J Pharmacol.* 2007 Mar; 150(6):738–49. [PubMed: 17293888]
31. Overwijk WW, Tsung A, Irvine KR, Parkhurst MR, Goletz TJ, Tsung K, et al. gp100/pmel 17 is a murine tumor rejection antigen: induction of “self”-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J Exp Med.* 1998 Jul 20; 188(2):277–86. [PubMed: 9670040]

32. Mendiratta SK, Thai G, Eslahi NK, Thull NM, Matar M, Bronte V, et al. Therapeutic tumor immunity induced by polyimmunization with melanoma antigens gp100 and TRP-2. *Cancer Res.* 2001 Feb 1; 61(3):859–63. [PubMed: 11221870]
33. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res.* 2007 Apr 1; 67(7):291–5.
34. Overwijk WW, Theoret MR, Finkelstein SE, Surman DR, de Jong LA, Vyth-Dreese FA, et al. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive CD8+ T cells. *J Exp Med.* 2003 Aug 18; 198(4):569–80. [PubMed: 12925674]
35. Dong H, Chen L. B7-H1 pathway and its role in the evasion of tumor immunity. *J Mol Med.* 2003; 81:281–7. [PubMed: 12721664]
36. Okazaki T, Iwai Y, Honjo T. New regulatory co-receptors: inducible co-stimulator and PD-1. *Curr Opin Immunol.* 2002; 14:779–82. [PubMed: 12413529]
37. Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A.* 2002 Sep 17; 99(19):12293–7. [PubMed: 12218188]
38. Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol.* 2005 Feb; 17(2):133–44. [PubMed: 15611321]
39. Andre F, Scharz NEC, Movassagh M, Flament C, Pautier P, Morice P, et al. Malignant effusions and immunogenic tumour-derived exosomes. *Lancet.* 2002 Jul 27; 360(9329):295–305. [PubMed: 12147373]
40. Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G, Villa A, et al. Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes. *Cancer Res.* 2006 Sep 15; 66(18):9290–8. [PubMed: 16982774]
41. Andreola G, Rivoltini L, Castelli C, Huber V, Perego P, Deho P, et al. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J Exp Med.* 2002 Mar 20; 195(10):1303–16. [PubMed: 12021310]

Figure 1. Expression of DC-HIL by B16F10 melanoma and characterization of DC-HIL-knocked-down cells

Expression of DC-HIL and other co-inhibitory ligands: (A) Total RNA was isolated from B16F10 melanoma cells untreated or treated with IFN- γ (1,000 U/ml) plus PMA (10 ng/ml) for 16 h or from BM-DC and examined by RT-PCR for mRNA expression of different co-inhibitory ligands and β -actin. PCR without cDNA (No cDNA) served as negative control. Characterization of DC-HIL-knocked-down B16F10 cells: B16F10 cells transfected with control lentivector (C-B16) or DC-HIL-targeted shRNA-lentivector (Kd-B16) were assessed for protein expression (B), *in vitro* proliferation (C), and subjected to cell cycle analysis (D). B, Whole cell extracts prepared from B16F10 transfectants were assayed by Western blotting for protein expression of DC-HIL or β -actin using UTX-103 anti-DC-HIL or anti- β -actin Ab. C, Synchronized B16F10 transfectants (1×10^4 cells/well) were allowed to grow *in vitro*, and proliferation measured by MTT assay (mean $\pm$ sd, n=3). D, After starving in serum-free media, B16F10 transfectants were stimulated for growth by FCS and labeled with BrdU, and then stained with APC-anti-BrdU Ab and 7-AAD (total DNA content), followed by flow cytometry. Data are shown as dot plots of BrdU vs. 7-AAD. All data are representative of at least 2 independent experiments.

Figure 2. Knockdown of DC-HIL expression reduces growth of B16F10 melanoma in syngeneic wild-type (but not nu/nu) mice

Control (C-B16) or DC-HIL-knockdown B16F10 cells (Kd-B16) were inoculated *s.c.* into the right flank of C57BL/6 mice (12 mice/group, A and B) or nu/nu mice (5 mice/group, C), and tumor volume measured daily. Survival rate of C57BL/6 mice injected with B16F10 transfectants is shown (B). The two transfectants were also mixed (each 2×10^5 cells) and examined for tumor growth in C57BL/6 mice ($n=10$) (D1) and survival rate (D2). Statistical analysis (Student's *t* test) of these two groups on days 13–21 indicates $p=2 \times 10^{-8}$ (A). ** ($p=1.5 \times 10^{-8}$) and *** ($p=0.001$) on day 24 document statistical significance of the mixed

cells' effect compared to C-B16 alone and Kd-B16 alone, respectively. Second experiment showed similar results.

Figure 3. Blockade of DC-HIL/SD-4 pathway leads to enhanced T cell immunogenicity

A, OVA-specific CD8⁺ T cells were stimulated by coculturing with OVA-pulsed/MMC-treated B16F10 transfectants and T cell activation assessed by production of IL-2 and IFN- γ . Statistical significance is denoted by * $p=0.05$ and ** $p=0.01$, as compared with production by T cells treated with C-B16 cells. **B**, Anti-OVA CTL were generated by immunization and subsequent *in vitro* stimulation and examined by flowcytometry for surface expression of SD-4 and CD69 (activation marker). **C**, OVA-specific CD8⁺ T cells were stimulated by OVA-pulsed/MMC-treated B16F10 melanoma in the absence (None) or presence of control IgG or anti-SD-4 mAb at different doses. T cell activation was assessed by IL-2 production. **D**, Anti-OVA CTL were allowed to kill target cells at varying E:T ratios. Target cells include OVA-pulsed Kd-B16 (• with solid lines), OVA-pulsed C-B16 (♣ with solid lines), untreated Kd-B16 (• with dashed lines), and untreated C-B16 cells (♣ with dashed lines). Statistical significance; *, $p=0.20$; **, $p=0.18$, and *** $p=0.58$. Cytotoxicity is expressed as % of lysis. All data are representative of 3 separate experiments.

Figure 4. DC-HIL knockdown enhances capacity to stimulate anti-TAA T cells

A, Anti-hgp100/TRP-2 CTL were stimulated by C-B16 or Kd-B16 melanoma cells and IFN- γ production measured. B, These CTL were cocultured with ^{51}Cr -labeled B16F10 transfectants or EL-4 as H-2-mismatched control, at varying E:T ratios. Statistical significance scores are denoted by * ($p=0.59$), ** ($p=0.69$), and *** ($p=0.77$). C, Two weeks after C57BL/6 mice ($n=7$) were tumor challenged with B16F10 transfectants, draining LN cells were prepared from treated mice and restimulated *in vitro* with γ -irradiated syngeneic spleen cells pulsed with hgp100 and TRP-2 peptides. Finally, IFN- γ -producing cells were counted by ELISPOT per 2×10^6 LN cells.

Figure 5. DC-HIL⁺ exosomes from B16F10 cells inhibit T cell activation triggered by TAA on melanoma

A, Exosomes prepared from C-B16 or Kd-B16 cells were examined by immunoblotting for expression of DC-HIL and gp100 (as a melanosomal marker). B, These exosomes were added to the coculture of activated CD8⁺ T cells (from pmel-1 TCR-transgenic mice) and MMC-treated B16F10 cells. T cell activation was measured by production of IL-2 (left) or IFN-γ (right panel) (mean ±sd, n=3. * $p > 0.001$).

Figure 6. Capacity of B16F10 transfectants to spread to lung and grow in the lung foci
C57BL/6 mice (n=6 for Exp 1 and n=5 for Exp 2) were given B16F10 transfectants by *i.v.* injection. 14 (Exp 1) or 11 (Exp 2) days post-injection, lungs were procured; weight, number of metastatic foci, total melanin content, and melanin content per focus were measured. Statistical significance (*p* value) is shown. Photographs of lungs from mice injected with B16F10 transfectants are shown on the right, with a scale bar of 50 mm. Images of some foci are enlarged.