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DC-HIL/Glycoprotein Nmb Promotes Growth of Melanoma in Mice by Inhibiting the Activation of Tumor-Reactive T Cells

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Abstract

DC-HIL/glycoprotein nmb (Gpnmb) expressed on antigen-presenting cells attenuates T-cell activation by binding to syndecan-4 (SD-4) on activated T cells. Because DC-HIL/Gpnmb is expressed abundantly by mouse and human melanoma lines, we postulated that melanoma-associated DC-HIL/Gpnmb exerts similar inhibitory function on melanoma-reactive T cells. We generated small interfering RNA–transfected B16F10 melanoma cells to completely knock down DC-HIL/Gpnmb expression, with no alteration in cell morphology, melanin synthesis, or MHC class I expression. This knockdown had no effect on B16F10 proliferation in vitro or entry into the cell cycle following growth stimulation, but it markedly reduced the growth of these cells in vivo following their s.c. 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 with controls) to activate melanoma-reactive T cells as documented in vitro and in mice. Whereas DC-HIL knockdown had no effect on susceptibility of melanoma to killing by cytotoxic T cells, blocking SD-4 function enhanced the reactivity of CD8+ T cells to melanoma-associated antigens on parental B16F10 cells. Using an assay examining the spread to the lung following i.v. injection, DC-HIL–knocked down cells produced lung foci at similar numbers compared with 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 the 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 antimelanoma immunotherapy.

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 CTLs that can lyse the tumor (1). On the other hand, tumors can use a variety of mechanisms that allow their escape from immunologic surveillance and destruction, including poorly expressed tumor-associated antigens (TAA), self-tolerance to TAA, downregulated MHC molecules, secretion of immunosuppressive cytokines [transforming growth factor, interleukin (IL)-10, and vascular endothelial growth factor], and deficient T-cell costimulation (2, 3).

T-cell activation results from signals delivered by antigen presenting cells (APC) to the antigen-specific T-cell receptor (TCR) and to costimulatory or coinhibitory receptors on T cells. The principal costimulatory signal is transmitted by CD80 or CD86 on APC to the CD28 receptor on T cells, which amplifies TCR activation signals (4). By contrast, coinhibitory molecules inhibiting TCR signals include the following: programmed cell death-1 (PD-1) and its ligands PD-L1 (B7-H1) and PD-L2 (5–7), B- and T-lymphocyte attenuator and herpesvirus entry mediator (4, 8), and Tim-3 ligand/Galectin-9 (on CD4+ T cells) and Tim-3 (on APC and Th1 effector cells; refs. 9–11). Like APC, tumors also may take advantage of coinhibitory 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 APCs (DC) led us to discover DC-HIL, a highly glyciosylated (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,

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and given a variety of names including glycoprotein nmb (Gpnmb; ref. 15), osteoactivin (16), and hematopoietic growth factor–inducible neurokinin-1 type (17). The extracellular domain of DC-HIL contains a cell-adhesion (RGD) motif, a proline-rich region, and an immunoglobulin-like polycystic kidney disease domain shared by polycystic kidney disease–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 antibody, thereby blocking the T-cell production of proinflammatory 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 coinhibitors 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 the growth of B16F10 melanoma in syngeneic mice by inhibiting the activation of tumor-reactive T cells, thereby making the DC-HIL/SD-4 pathway a potentially useful target for antitumor melanoma immunotherapy.

Materials and Methods

Mice and cell culture

Female C57BL/6 mice (5- to 8-wk-old) were purchased from Harlan Breeders; nu/nu and pmel-1 TCR transgenic mice [B6.Cg- Thy1a/Cy Tg(TcraTcrb)8Rest/J] were from Jackson Laboratory; and C57BL/6 and pmel-1 TCR transgenic mice 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 the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FCS.

Reverse transcription-PCR analysis

Total RNA (1 μg) isolated from B16F10 cells or DCs was converted to cDNA by reverse transcriptase (Life Technologies, Inc.; ref. 22). An aliquot (typically 5%) was used for PCR amplification (22) using the primers: for DC-HIL, 5′-CTCGCAAGCCTCGTGTGCTG-3′ and 5′-CCTGTCGGGAACCTGAGATGC-3′; for PD-L1, 5′-CCCACTCGGCAAATCAACAGG-3′ and 5′-CAACGCCACATTTCCTCACATCTA-3′; for PD-L2, 5′-CACCGTCAAGCCCTAAAGAAGT-3′ and 5′-GGGTTCTGTGTGTTTGGTTGT-3′; for herpesvirus entry mediator, 5′-CATCTCTGCAACGGGCTCAT-3′ and 5′-GCTATCCCAACTCCACTACACA-3′; for Tim-3, 5′-TGCCAAAGTCTAGTGTTCAGTGC-3′ and 5′-CTCTCCGTGGTTAGGGTTCTGT-3′; or for β-actin, 5′-GAGCGGAAAATGTGCTGATGGATT-3′ and 5′-GAAGTATGTTCGTGATGCCC-3′. Following 30 cycles of amplification, PCR products were separated electrophoretically on 1% agarose gel.

Plasmid vectors

We constructed a small interfering RNA (siRNA)–expressing lentivirus consisting of DC-HIL–targeted short hairpin RNA (shRNA; 58 base oligonucleotides, the sense strand, 5′-GATCGG-GGAACCTGTGTGCTGAGTCT-CGAAAGATCTCATCAGACAACTGCC-3′; and the antisense strand, 5′-AATTCAAAA-GGAACCTGTGTGCTGAGTCT-TTCG-AGATCTCATCAGACAACTGCC-3′, in which the italic nucleotides are BamHI site in the sense strand and EcoRI in the antisense strand synthesized by Integrated DNA Technologies and ligated to the downstream of H1 RNA polymerase III promoter in pSIF1-H1-copGFP shRNA lentivector (System Biosciences) that contains a cytomegalovirus-driven fluorescent copedep green fluorescent protein (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 × 10⁷) were infected with control or DC-HIL-shRNA lentivector at a multiplicity of infection of 20. The next day, GFP+ cells were enriched repeatedly by fluorescence-activated cell sorting (FACS) until >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 minutes, followed by centrifugation for 20 minutes at 10,000×g (24). An aliquot (40 μg) of extract was applied to SDS/4% to 15% gradient PAGE, followed by immunoblotting using UTX-103 rabbit anti-DC-HIL monoclonal antibody (mAb; 2 μg/mL; ref. 25) or anti–β-actin antibody (Abcam). For examining the 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.). Color was developed by horseradish peroxidase–secondary antibody (1:10,000 dilution, Jackson ImmunoResearch Laboratories, Inc.) for 1 hour and enhanced chemiluminescence plus system (Amersham Pharmacia Biotech).

Flow cytometry

To analyze surface expression, B16F10 cells (1 × 10⁷) were incubated with UTX-103 mAb, biotinylated anti-H-2Db (eBioscience), or isotype control antibody (each 5 μg/mL). To measure the expression of H-2Kb molecule bound to ovalbumin (OVA) peptide, B16 F10 cells were preincubated with 2 μg/mL OVA257-264 (SIINFEKL) for 2 hours before staining with 5 μg/mL phycoerythrin (PE)–conjugated anti–H-2Kb (Kb-SIINFEKL, eBioscience). After extensive washing, cells were labeled fluorescently with secondary antibody [5 μg/mL PE- or FITC-anti-rabbit IgG (Jackson ImmunoResearch Laborato
Laboratories, Inc.) or 5 μg/mL PE-streptavidin (BD Biosciences). 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 hours in serum-free media to synchronize cell growth. Thereafter, 1 × 10^4 cells per well were seeded in 96-well plates (in triplicate) and cultured in complete DMEM. Cell growth was determined each day by adding 10 μL MTT Reagent (from ATCC). Cells were then incubated at 37°C for 2 hours, and 100 μL Detergent Reagent (ATCC) were added and left at room temperature in the dark for 2 hours. Absorbance was determined at 595 nm.

**Cell cycle analysis**

B16F10 melanoma cells were plated on a Petri dish (5 × 10^5 cells/dish) and starved for 24 hours in serum-free media to synchronize cell growth. Thereafter, 1 × 10^4 cells per well were seeded in a 96-well plate (in triplicate) and cultured in complete DMEM. Cell growth was determined each day by adding 10 μL MTT Reagent (from ATCC). Cells were then incubated at 37°C for 2 hours, and 100 μL Detergent Reagent (ATCC) were added and left at room temperature in the dark for 2 hours. Absorbance was determined at 595 nm.

**In vivo tumor growth**

C57BL/6 or nu/nu mice were inoculated with control or knockdown B16F10 transfectants by s.c. 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 × D × 0.5), in which d and D are the minor and major diameters, respectively (26). For ethical reasons, animals were sacrificed when tumors grew to a volume of >5 cm^3.

**Exosome preparation**

Culture supernatant was harvested from 72-hour culture of confluent melanoma cells (C-B16 and Kd-B16). The supernatant was centrifuged at 5,000× g for 30 minutes at 4°C to remove cell debris, and the obtained supernatant was then ultracentrifugated at 100,000× g for 6 hours 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), were seeded at a density of 2 × 10^6 cells per well in 96-well microplates (in triplicate) and cocultured with B16F10 transfectants (2 × 10^4 cells/well) pulsed with 2 μg/mL OVA257-264 for 3 hours, then subsequently treated with mitomycin C (MMC, 25 ng/mL) for 1 hour. After culturing for 2 days, 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). Seventeen days postimmunization, cells were prepared from spleen and lymph nodes (LN) of the mice, which were then stimulated in vitro with γ-irradiated (3,000 Gy) syngeneic spleen cells that were pulsed with the peptide mixture (2 μg/mL hgp100 and 2 μg/mL TRP-2). After 10 days in culture, CD8+ T cells were isolated and similarly cocultured with MMC-treated B16F10 transfectants for 2 days. 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. Fourteen days postinoculation, draining LN cells were prepared and cocultured with γ-irradiated syngeneic spleen cells pulsed with the TAA peptide mixture (each 2 μg/mL) for 7 days. Thereafter, cells were extensively washed, adjusted to 2 × 10^6 cells/mL, 2-fold serially diluted, and seeded on a 96-well polyvinylidene difluoride membrane ELISPOT plate (MultiScreenHTSIP Filter plate). The number of IFN-γ-producing cells was counted and calculated per 2 × 10^5 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 μg/mL hgp100 peptide (EGSRNQDWL, from ANASPEC) and cocultured with MMC-treated B16F10 cells (2 × 10^5/well) in the presence of indicated doses of exosomes for 2 days. IL-2 and IFN-γ production was measured.

**Cytotoxicity assay**

B16F10 or EL-4 tumor cells (1 × 10^5) were preincubated with or without 2 μg/mL OVA257-264 or TAA peptide mixture (each 2 μg/mL) for 3 hours and then labeled with 100 μCi of Na^{51}Cr for 1 hour. OVA-specific or TAA-immunized T cells were added to the labeled tumor cells (1 × 10^5/well) at varying effector-target ratios and were incubated for 4 hours. Specific Na^{51}Cr release was determined as previously described (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 days postinjection, and their total weight, number of metastatic foci, and melanin content were examined (29). Melanin was quantified in lungs as previously described (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 coinhibitory ligands

To determine the expression profile of coinhibitory ligand genes by melanoma cells, we used reverse transcription-PCR (RT-PCR) to analyze mRNA expression of DC-HIL, PD-L1, PD-L2, herpesvirus entry mediator, and Tim-3. Bone marrow–derived DCs served as positive control because 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 bone marrow–derived DC (Fig. 1A); PD-L1 was present but at a much lower level, and the other coinhibitory ligands not at all. IFN-γ treatment did not change the 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 five different shRNA oligonucleotides and inserted each into a lentivector. The efficacy of each shRNA to knock down DC-HIL expression was assessed by the reduction in protein expression. The shRNA showing highest efficacy (data not shown) was used to generate B16F10 cells knocked down transfectant. To avoid clonal effects, infected B16F10 cells were enriched by repeated FACS instead of cloning by limiting dilution. One batch of infected B16F10 cells was assayed by Western blotting for DC-HIL protein (Fig. 1B). Compared with control B16F10 cells (called 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 Fig. S1). There were no morphologic changes in Kd-B16 cells, and no significant differences in the expression of MHC class I molecules H-2Dβ and OVA-associated H-2Kβ 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 hours; cell cycle analysis was performed using BrdUrd 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 the 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 the mice,
and tumor volume was 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 versus $1.61 \pm 0.68 \text{ cm}^3$ for Kd-B16 at 24 d 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 with controls (Fig. 2C). We also questioned whether concomitant inoculation of C-B16 and Kd-B16 cells (mixed in equal numbers) suppresses the growth of s.c. injected C-B16 tumor (Fig. 2D). Indeed, the growth of the mixed cells was reduced significantly throughout the experiment, compared with 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 with 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 Fig. S4B). Growth of NS-B16 tumor was similar to C-B16 by day 20 and thereafter slightly slower than C-B16 (Supplementary Fig. S4C). We then compared effects of siRNA targeting DC-HIL versus 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 $\beta$-actin and DC-HIL levels were unchanged (Supplementary Fig. S4A and B). 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 Fig. 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 the 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 effect of knocking down DC-HIL on the immunogenicity of T cells for B16F10 melanoma cells. Because B16F10 melanoma is inherently weak immunogenically, we used OVA as a surrogate 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.

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**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 was measured daily. Survival rate of C57BL/6 mice inoculated with the two transfectants is shown (B). The two transfectants were also mixed (each 2 × 10⁵ 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 to 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 with C-B16 alone and Kd-B16 alone, respectively. Second experiment showed similar results.
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 were finally examined by flow cytometry for SD-4 expression (Fig. 3B). All CTL expressed the CD69 activation marker, and half of CD69+ CTL coexpressed SD-4. To examine the involvement of SD-4 in T-cell immunogenicity by melanoma cells, we assayed the 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 the 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 seem to be similarly sensitive to CTL cytotoxicity.

To ascertain the effect of knocking down DC-HIL, we used hgp100 and TRP-2 antigen that have been used as TAA in antimelanoma immunotherapy (31, 32). CD8+ CTL were prepared by in vitro restimulation of LN cells isolated from C57BL per six mice immunized with the two peptides, and were finally cocultured with C-B16 or Kd-B16 cells (Fig. 4). Consistent with results of studies using OVA peptide, CTLs

![Figure 3](image1.png) 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 was assessed by production of IL-2 and IFN-\( \gamma \). Statistical significance is \( P = 0.05 \left( \ast \right) \) and \( P = 0.01 \left( \ast \ast \right) \), compared with the 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 flow cytometry 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 effector-target ratios (E/T). Target cells include OVA-pulsed Kd-B16 (● with solid lines), OVA-pulsed C-B16 (Δ with solid lines), nontreated Kd-B16 (● with dashed lines), and nontreated C-B16 cells (Δ with dashed lines). Statistical significance scores are \( P = 0.20 \left( \ast \right) \), \( P = 0.18 \left( \ast \ast \right) \), and \( P = 0.58 \left( \ast \ast \ast \right) \). Cytotoxicity is expressed as % of lysis. All data are representative of three separate experiments.

![Figure 4](image2.png) DC-HIL knockdown enhances the 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-\( \gamma \) production was measured. B, these CTL were cocultured with \( ^{51} \)Cr-labeled B16F10 transfectants or EL-4 as H-2–mismatched control, at varying effector-target (E/T) ratios. Statistical significance scores are \( P = 0.59 \left( \ast \right) \), \( P = 0.69 \left( \ast \ast \right) \), and \( P = 0.77 \left( \ast \ast \ast \right) \). C, 2 wk 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 \( \gamma \)-irradiated syngeneic spleen cells pulsed with hgp100 and TRP-2 peptides. Finally, IFN-\( \gamma \)–producing cells were counted by ELISPOT per \( 2 \times 10^6 \) LN cells.
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 the 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 antigen**

Because 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 μg/mL) from C-B16 cells led to 80% reduction in IL-2 production by pmel-1 T cells (50% with 100 μg/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 the lung, but it reduces growth of melanoma within lung foci**

We next examined the effect of DC-HIL knockdown on the dissemination of B16F10 melanoma to the lung following i.v. injection into syngeneic hosts (Fig. 6). Two weeks after infusing B16F10 transfectants into C57BL/6 mice through the tail vein, mice were sacrificed and lungs were assayed for weight, number of foci, and melanin content (exp. 1). Comparing the lungs of mice injected with C-B16 cells to those injected with Kd-B16 cells, the latter were lighter (0.21 ± 0.02 g versus 0.20 ± 0.02 g, P = 0.23) and contained more lung foci (2,091 ± 240 versus 2,333 ± 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 μg versus 611 ± 112 μg, P = 0.11) and melanin per focus was significantly less (0.40 ± 0.11 versus 0.27 ± 0.05, P = 0.03). A second experiment (exp. 2) reproduced the latter finding (0.68 ± 0.12 versus 0.43 ± 0.21, P = 0.04). These results indicate that the effect of knocking down DC-HIL in B16F10 cells is manifest, not in the capacity for establishment in lung following i.v. injection, but in their potential for growth within lung foci.

**Discussion**

To determine whether melanoma-associated DC-HIL inhibits the 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

![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). Columns, mean (n = 3); bars, SD. *P < 0.001.](image-url)
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 s.c. and i.v. 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 because: (a) knocking down DC-HIL in B16F10 cells enhanced the capacity of these cells to stimulate melanoma-reactive T cells; (b) IFN-\(\gamma\)-secreting effector T cells were generated more in mice challenged with the knocked down cells; (c) SD-4 is expressed by melanoma-reactive CTL; and (d) parental B16F10 melanoma enhanced activation of CTL when SD-4 function is blocked. By contrast, we were unable to show any significant effect of DC-HIL on susceptibility to CTL-mediated lysis or on the ability of melanoma to become established in lung following i.v. injection. Moreover, the inhibitory function of DC-HIL may be delivered to lymphoid organs distal to the melanoma site through exosomes. Altogether, our findings indicate that DC-HIL confers on melanoma the ability to inhibit the activation of melanoma-reactive T cells, thereby compromising optimal antitumor immunity.

The Gpnmb/DC-HIL gene was identified first by Weterman and colleagues (15) using subtractive cDNA cloning between highly and lowly metastatic human melanoma cell lines. 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 article (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. However, they also reported that two of their three 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: (a) mouse versus human melanoma cell lines; (b) bulk versus cloned transfectants; (c) Gpnmb protein expression was not examined in their BLM transfectants; (d) knockdown (loss-of-function) strategy by us versus transgenic expression (gain-of-function) by them; and (e) i.v. injection of B16F10 transfectants into syngeneic immunocompetent mice by us versus 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 coinhibitors of T-cell activation, DC-HIL most closely resembles PD-L1 in its function (19). PD-L1 is expressed by different cancers and is 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-\(\gamma\) 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 TCR 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 overexpressing 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 antigen pmel-17 (mouse homologue 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; ref. 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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