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*J Immunol* 2012; 188:4297-4304; Prepublished online 9 March 2012;
doi: 10.4049/jimmunol.1102343
http://www.jimmunol.org/content/188/9/4297

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/03/13/jimmunol.1102343.DC1

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Enhancing Immunostimulatory Function of Human Embryonic Stem Cell-Derived Dendritic Cells by CD1d Overexpression

Jieming Zeng,* Mohammad Shahbazi,**† Chunxiao Wu,* Han Chong Toh,‡ and Shu Wang*†

Human embryonic stem cell-derived dendritic cells (hESC-DCs) may potentially provide a platform to generate “off-the-shelf” therapeutic cancer vaccines. To apply hESC-DCs for cancer immunotherapy in a semiallogeneic setting, it is crucial for these cells to “jump-start” adaptive antitumor immunity before their elimination by host allorejection. In this study, we investigated whether CD1d upregulation in hESC-DCs may exploit invariant NKT (iNKT) cell adjuvant activity and boost antitumor immunity. Using a baculoviral vector carrying the CD1d gene, we produced CD1d-overexpressing hESC-DCs and demonstrated that the upregulated CD1d was functional in presenting α-galactosylceramide for iNKT cell expansion. Pulsed with melanoma Ag recognized by T cell 1 peptide, the CD1d-overexpressing hESC-DCs displayed enhanced capability to prime CD8+ T cells without relying on α-galactosylceramide loading. Blocking the CD1d with Ab reduced the immunogenicity, suggesting the importance of hESC-DC and iNKT cell interaction in this context. The CD1d-overexpressing hESC-DCs also induced a proinflammatory cytokine profile that may favor the T cell priming. Moreover, a similar immunostimulatory effect was observed when the CD1d upregulation strategy was applied in human monocyte-derived dendritic cells. Therefore, our study suggests that the upregulation of CD1d in hESC-DCs provides a novel strategy to enhance their immunogenicity. This approach holds potential for advancing the application of hESC-DCs into human cancer immunotherapy. *The Journal of Immunology, 2012, 188: 4297–4304.

Dendritic cells (DCs), known as professional APCs, are one of the attractive manipulation targets in cancer immunotherapy because of their ability to initiate and regulate antitumor T cell immunity (1). With approval of the first DC-based vaccine, Dendreon’s Provenge, autologous DC-based therapy is being established as a new modality for cancer treatment (2). However, the preparation of autologous cell therapeutics is expensive for patients and technically demanding for clinicians, not to mention the difficulty involved with large-scale industrial production (2).

Human embryonic stem cells (hESCs) are capable of differentiating into functional DCs, thus providing an unlimited novel DC source (3–5). The use of hESC-derived DCs (hESC-DCs) may bypass the logistic and quality issues inherent in using a patient’s DCs. However, to apply the hESC-DCs as cancer vaccine, it is crucial for them to “jump-start” the tumor Ag-specific CTL response before their elimination by host allorejection. In this study, we investigated whether CD1d upregulation in hESC-DCs may exploit invariant NKT (iNKT) cell adjuvant activity and boost antitumor immunity. Using a baculoviral vector carrying the CD1d gene, we produced CD1d-overexpressing hESC-DCs and demonstrated that the upregulated CD1d was functional in presenting α-galactosylceramide for iNKT cell expansion. Pulsed with melanoma Ag recognized by T cell 1 peptide, the CD1d-overexpressing hESC-DCs displayed enhanced capability to prime CD8+ T cells without relying on α-galactosylceramide loading. Blocking the CD1d with Ab reduced the immunogenicity, suggesting the importance of hESC-DC and iNKT cell interaction in this context. The CD1d-overexpressing hESC-DCs also induced a proinflammatory cytokine profile that may favor the T cell priming. Moreover, a similar immunostimulatory effect was observed when the CD1d upregulation strategy was applied in human monocyte-derived dendritic cells. Therefore, our study suggests that the upregulation of CD1d in hESC-DCs provides a novel strategy to enhance their immunogenicity. This approach holds potential for advancing the application of hESC-DCs into human cancer immunotherapy. *The Journal of Immunology, 2012, 188: 4297–4304.
one strategy to manipulate the iNKT cell response is to use synthetic iNKT cell agonists (18). Through structure-guided design, these novel iNKT cell agonists may minimize the side effects associated with iNKT cell overstimulation by strong agonists (e.g., α-GC), while inducing differential cytokine production. A pure Th1- or Th2-like response can be generated by these synthetic α-GC analogs, which may be attributed to their different affinity for the iNKT TCRs, although the detailed mechanisms remain unclear.

In addition to modifying the affinity of iNKT cell ligands, manipulation of Ag abundance on APCs through the regulation of CD1d expression may affect the nature of the cytokine response in iNKT cells. A study on murine macrophages showed that the microbial infection or microbial products can upregulate the expression level of CD1d and activate iNKT cells (19). In studies on DCs, viral infection is capable of downregulating CD1d expression to evade recognition by iNKT cells (20–22). These findings suggest that the regulation of CD1d expression on the DC surface may present a novel strategy to modulate iNKT cell activation and its immunological outcome. In the present study, we examined whether the enforced upregulation of CD1d in hESC-DCs can be used to exploit iNKT cell adjuvant activity and assist the priming of CD8+ T cells against tumor Ag.

Materials and Methods

Cell culture

An hESC line, HES-1 (ES Cell International, Singapore) was maintained on mouse embryonic fibroblasts, as described before (23). Two other hESC lines, H1 and H9 (WiCell Research Institute, Madison, WI), were maintained on Matrigel-coated (BD Biosciences, San Jose, CA) six-well plates using mTeSR1 medium (STEMCELL Technologies, Vancouver, Canada), according to the manufacturer’s technical manual. A mouse bone marrow stromal cell line OP9 (American Type Culture Collection [ATCC], Manassas, VA) was maintained with α-MEM (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (HyClone, Logan, UT).

Human DCs were generated from hESCs based on a three-step protocol, as previously described (4). In brief, the myeloid precursors were induced from hESCs by coculturing with OP9 cells for 9 d and further expanded in the presence of GM-CSF (PeproTech, Rocky Hill, NJ) for 10 d. DCs were generated from those myeloid precursors by culturing in DC-differentiation medium composed of StemSpan serum-free expansion medium (STEMCELL Technologies, Seattle, WA), supplemented with lipid mixture 1 (Sigma-Aldrich, St Louis, MO), 100 ng/ml GM-CSF, and 100 ng/ml IL-4 (PeproTech) for 8–12 d. The generated DCs were then purified by staining with allophycocyanin mouse anti-human CD209 Ab (BD Biosciences) and sorting with a FACSARia flow cytometer (BD Biosciences). To produce human monocyte-derived DCs (moDCs), frozen human PBMCs (STEMCELL Technologies) were thawed and cultured on T75 for 2 h. The plastic-adherent cells were differentiated in the DC-differentiation medium for 6 d to generate moDCs. Frozen human PBMCs, HLA-A2+ PBMCs, or peripheral blood mononuclear cells from healthy donors were cultured in DC-differentiation medium (STEMCELL Technologies) and were thawed and cultured in complete RPMI 1640, which is composed of RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated human serum AB (Gemini Bio-Products, West Sacramento, CA), 2 mM l- glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), and 0.1 mM 2-ME (Invitrogen). The human PBLCs were generated from PBMCs via removing the monocytes by plastic adherence.

A human melanoma cell line Malme-3M (ATCC) was cultured in IMDM (Invitrogen) with 20% FBS. A human colorectal adenocarcinoma cell line SW-480 (ATCC) was maintained in Leibovitz’s L-15 Medium (Invitrogen) with 10% FBS.

Baculovirus preparation and transduction

A pFastBac1 plasmid (Invitrogen) carrying the CD1d gene was used to produce baculoviral vector. To construct the plasmid, the human CMV promoter immediately early gene promoter and enhancer (CMV promoter) was amplified by PCR and placed between the BamHI and EcoRI cloning sites of pFastBac1. Then, the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) was PCR amplified from pPsbCMV-eGFP-WPRE (kindly provided by Professor H. Büeler, University of Zurich, Zurich, Switzerland) and inserted between the SpeI and XbaI sites of pFastBac1. The coding sequence of human CD1d was cloned from pORF9-hCD1d (InvivoGen, San Diego, CA) through PCR to include the Kozak sequence upstream of its start codon and the Sall and Spel restriction sites at its terminus. Two of these two sites were used to insert the CD1d gene. Baculovirus carrying the CD1d gene (BVCD1d) was produced using the above-described plasmid and propagated in Sf9 cells according to the Bac-to-Bac Baculovirus Expression System manual (Invitrogen). A control baculovirus carrying the BacPAK6 viral gene (BV) was produced using BacPAK6 viral DNA (Clontech, Mountain View, CA) that does not express transgene in mammalian cells. Baculoviruses were purified as described previously (24). Their infectious titers (PFUs) were determined by plaque assay using Sf9 cells.

For baculoviral transduction, the human DCs were resuspended in the DC-differentiation medium at a density of 107/100 μl. The baculoviral vectors suspended in 100 μl PBS were then added to transduce DCs for 4 h. A multiplicity of infection (MOI) of 100 was used unless indicated otherwise. The DCs were washed twice and incubated for 1–2 d before use in further experiments. To detect CD1d expression, the transduced DCs were stained by PE mouse anti-human CD1d Ab (BD Biosciences) and analyzed using a FACSARia flow cytometer (BD Biosciences).

Measurement of allostimulatory function of human DCs

To measure the allostimulatory function of DCs, frozen human peripheral blood pan-T cells were thawed and used as responders. The pan-T cells were first resuspended in PBS containing 5% heat-inactivated FBS (107 cells/ml). Cells were then cultured in 24-well plates with 100 ng/ml GM-CSF and 100 ng/ml IL-4 (PeproTech) for 8–12 d. After washing three times with PBS containing 5% heat-inactivated FBS, the cells were resuspended in complete RPMI 1640 at a density of 107/ml. To set up the allogeneic stimulation assay, 2 × 105 CFSE-labeled pan-T cells were cocultured with DCs suspended in complete RPMI 1640. A polyclonal activator, mitogenic lectin PHA (Sigma-Aldrich), which stimulates T cell proliferation, was used as a positive control. After a 5-d incubation, the cells were stained with allophycocyanin mouse anti-human CD3 Ab (BD Biosciences), and T cell proliferation was measured by CFSE dilution using a FACSARia flow cytometer.

Detection of iNKT cell expansion, CTL expansion, CTL activity, and cytokine production

To analyze the effect of CD1d upregulation in DCs on the expansion of human iNKT cells, hESC-DCs were transduced by BCVCD1d. One day after transduction, the DCs were pulsed with 100 ng/ml α-GC (Axxora, Lausen, Switzerland) for 24 h. Two days after transduction, 107 α-GC–pulsed DCs were washed and cocultured with 105 PBLS or pan-T cells in complete RPMI 1640 containing 100 ng/ml α-GC. In some experiments, α-GC–pulsed DCs were used without further addition of free α-GC into the culture medium. To detect iNKT cells, the samples were collected 7 d after coculture, stained with allophyocyanin mouse anti-human CD3 (BD Biosciences), PE anti-Vε2 (Serotec, Oxford, UK), and FITC anti-Vβ11 TCR (Beckman Coulter), and analyzed using a FACSARia flow cytometer. For each assay, 500,000 events were collected.

To stimulate Ag-specific CTL responses, HLA-A2–H1-derived DCs (H1.DCs) or moDCs were used to present the HLA-A2-restricted epitope MART-126–35 (a27L) (ELA4G17TV; melanoma Ag recognized by T cell 1 [MART-1] peptide; ProImmune, Oxford, U.K.). The DCs were transduced by BVCD1d and pulsed or not with 100 ng/ml α-GC for 24 h, 1 d after transduction. Two days after transduction, the DCs were pulsed with 10 μg/ml MART-1 peptide for 4 h. After washing, 105 DCs were cocultured with 105 HLA-A2+ PBLS in complete RPMI 1640. To detect tumor Ag-specific CTLS, the samples were collected 9 d after coculture, stained with allophycocyanin mouse anti-human CD3, FITC-labeled anti-CD8A (ProImmune), and R-PE–labeled A*0201/ELA4G17TV Pentamer (ProImmune), and analyzed using a FACSARia. In some experiments, the CD1d-overexpressing human DCs were pretreated with anti-CD1d blocking Ab (eBioscience, San Diego, CA) or its isotype control (eBioscience) before coculture with PBLS. To quantify the effect of glycolipid biosynthesis inhibition on T cell priming, the BVCD1d-transduced human DCs were treated with 20 μM DL-threo-1-Phenyl-2-halmitono-3-morpholin-1-propanol (PMPm; Sigma-Aldrich) or 150 μM N-(α-Butyl)dideoxygalactononiririmycin (NB-DGJ; Merck KGaA, Darmstadt, Germany) for 24 h, 1 d after transduction. In other experiments, the cocultures were restimulated with 105 MART-1 peptide-pulsed DCs on day 9 of coculture and analyzed 1 wk after restimulation. To measure tumor Ag-specific CTL activity, the cocultures were stimulated for the third time on day 7 after the second stimulation with 105 MART-1 peptide-pulsed DCs for 48 h and used as targets for lytic assays. A luminescence-based assay was used to measure tumor Ag-specific CTL activity. In brief, the tumor cells Malme-3M or SW-480 were labeled with luciferase by transfection with a plasmid containing a luciferase
transfection, 10^5 tumor cells were used as targets and incubated with the effectors described above at the desired E:T ratios. After a 6-h incubation, D-Luciferin (Caliper Life Sciences, Hopkinton, MA) was added to a final concentration of 150 μg/ml. Ten minutes later, luminescence was measured, and the percentage of specific lysis was calculated using the relative light unit (RLU) of the experimental sample (with effectors, RLU_{exp}) as follows: percentage of specific lysis = \[ 1 - \frac{RLU_{exp} - RLU_{ basal}}{RLU_{ media} - RLU_{ basal}} \times 100. \]

To study cytokines secreted during coculture of DCs and PBLs, 10^5 H1.DCs were transduced by baculoviral vectors. The transduced DCs were then incubated with 10^6 PBLs in 300 μl complete RPMI 1640. Three days later, the supernatants were collected, and cytokine concentration was measured with Cytometric Bead Array (CBA) assay using the CBA Human Soluble Protein Master Buffer Kit and the CBA Flex Sets that detect INF-γ, IL-4, and TNF (BD Biosciences). In some experiments, CD1d-overexpressing H1.DCs were pretreated with anti-CD1d blocking Ab or its isotype control before coculturing with PBLs.

**Results**

**CD1d upregulation in hESC-DCs by baculoviral transduction**

To have a reliable DC source, three hESC lines (H1, H9, and HES-1) were used to generate human DCs (Supplemental Figs. 1, 2). Flow cytometry analysis demonstrated that these hESC-DCs expressed typical DC surface markers, including CD209, CD11c, CD86, and HLA-DR (Supplemental Fig. 2). Because the H1 line was HLA-A2^+ and more reliable and consistent in terms of DC yield, the H1.DCs were purified (Supplemental Fig. 1E–G) and used in the following experiments.

To enforce the upregulation of CD1d in the hESC-DCs, the transduction efficiency of baculoviral vector was evaluated. Using BVCD1d, a baculoviral vector carrying a CD1d expression cassette, we observed that up to 51% of the H1.DCs displayed CD1d upregulation 2 d after transduction at an MOI of 100 (Fig. 1A). The CD1d expression level on H1.DCs did not change after transduction using BV, a baculoviral vector without a mammalian gene expression cassette (Fig. 1A), indicating that baculoviral transduction alone is unable to upregulate CD1d. Transduction with an MOI of 500 failed to increase CD1d expression further (Fig. 1A), possible as a result of cytotoxicity associated with a high dose of baculovirus.

To assess the possible effects of baculoviral transduction on phenotype and function of the hESC-DCs, we used BV to transduce the H1.DCs at different MOIs. At an MOI of 100, the baculoviral transduction did not significantly change the cell surface expression of CD40, CD83, or CD86, whereas the expression of HLA-DR was slightly upregulated (Fig. 1B). However, the expression of these surface molecules was apparently reduced when the MOI was increased to 500 (Fig. 1B). DC function after baculoviral transduction was assessed by their ability to stimulate allogeneic T cell proliferation using a CFSE-based assay, wherein PHA was used as a positive control. At an MOI of 100, the baculovirus-transduced H1.DCs were able to stimulate allogeneic T cell proliferation in a dose-response manner similar to that induced by the mock-transduced H1.DCs (Fig. 1C). Again, at an MOI of 500, the allostimulatory function of baculovirus-transduced H1.DCs was obviously reduced (Fig. 1C). These results indicate that a suitable dose of baculovirus should be selected to minimize the effect of baculoviral transduction on hESC-DC phenotype and function. Thus, an MOI of 100 was used for transduction in the following experiments.

**CD1d upregulation in hESC-DCs is functional in promoting human iNKT cell expansion**

To study whether the CD1d molecules overexpressed in hESC-DCs are functional, BVCD1d-transduced H1.DCs were evaluated for their ability to present α-GC for human iNKT cell expansion. As shown by using healthy human PBLs as responders, the CD1d-overexpressing H1.DCs were more efficient than the mock-transduced H1.DCs and the BV-transduced H1.DCs in inducing iNKT cell expansion (Fig. 2A, Supplemental Fig. 3A). To exclude the effects of possible α-GC presentation by B cells and the residual monocytes in the PBLs, pure human peripheral blood monocytes were used as responders, and a similar result was obtained (Fig. 2A, Supplemental Fig. 3A). Noticeably, the induction of iNKT cell expansion by CD1d-overexpressing H1.DCs depended on the presence of α-GC, whereas there was no obvious iNKT cell expansion in the absence of α-GC (Fig. 2B, Supplemental Fig. 3B). Furthermore, compared with unmodified autologous moDCs, the CD1d-overexpressing H1.DCs were more effective in promoting iNKT cell expansion (Fig. 2C, Supplemental Fig. 3C). To rule out the possible effects of free α-GC, the...
α-GC–pulsed H1.DCs were used without further addition of α-GC to the coculture. The result showed that the α-GC–pulsed CD1d-overexpressing H1.DCs were effective enough in inducing iNKT cell expansion and outperformed the α-GC–pulsed BV-transduced H1.DCs (Fig. 2D).

CD1d upregulation in hESC-DCs enhances priming of CD8\(^+\) T cells against tumor Ag without relying on α-GC loading

To investigate the effect of CD1d upregulation in H1.DCs on their ability to prime tumor Ag-specific CTL responses, the BVCD1d-transduced H1.DCs were pulsed with α-GC and MART-1 peptide, an immunodominant peptide derived from the melanoma Ag MART-1. The pulsed H1.DCs were then cocultured with HLA-A2+ PBLs to induce the CTL response. After coculture for 9 d, pentamer staining was performed to identify the MART-1 peptide-specific CD8\(^+\) T cells (Fig. 3). The results showed that without α-GC loading, the CD1d-overexpressing H1.DCs displayed significantly improved efficacy in inducing specific expansion of CD8\(^+\) T cells compared with those DCs without CD1d upregulation, such as the BV-transduced H1.DCs (p < 0.009) or the autologous moDCs (p < 0.04) (Fig. 3B). However, with α-GC loading, there was no obvious beneficial effect in priming the Ag-specific CD8\(^+\) T cells when the mock-transduced H1.DCs, BV-transduced H1.DCs, and autologous moDCs were used (Fig. 3B). It was also noted that, with α-GC loading, the priming ability of CD1d-overexpressing H1.DCs was even significantly decreased compared with their counterparts without α-GC loading (p < 0.04) (Fig. 3B). Without loading the MART-1 peptide, there was no observable specific expansion of CD8\(^+\) T cells by all of the DCs (Fig. 3B), further confirming the specificity of the CD8\(^+\) T priming. These results suggest that CD1d upregulation alone is able to enhance the immunogenicity of the hESC-DCs, and this effect does not depend on the loading of exogenous iNKT cell ligand.

Restimulation with the MART-1 peptide-loaded H1.DCs further expanded the MART-1 peptide-specific CD8\(^+\) T cells, and the expansion was more obvious in the coculture that was initially primed by CD1d-overexpressing H1.DCs (Fig. 4A). Moreover, after the third stimulation with Ag-loaded H1.DCs, the MART-1 peptide-specific CD8\(^+\) T cells were able to kill the HLA-A2\(^+\) and MART-1\(^+\) melanoma cell line Malme-3M but not the HLA-A2\(^+\) and MART-1\(^+\) tumor cell line SW-480 (Fig. 4B), demonstrating that these T cells were functional in lysing the target tumor cells specifically.

CD1d-upregulation strategy can be applied to human moDCs for enhancing T cell priming

Considering the importance of autologous moDCs as one of the useful cell sources in current DC-based immunotherapy, we explored whether the CD1d-upregulation strategy could also be applied to moDCs for enhancing T cell priming. Our result showed that after transduction with BVCD1d at an MOI of 100, up to 60% of moDCs expressed CD1d on day 2 (Fig. 5A). Baculoviral transduction alone did not upregulate CD1d expression, as demonstrated in moDCs transduced by BV (Fig. 5A). Similar to the findings obtained from H1.DCs, the α-GC–pulsed CD1d-overexpressing moDCs were obviously superior in expanding iNKT cells to the α-GC–pulsed BV-transduced moDCs (Fig. 5B). Without α-GC...
MART-1 peptide for 4 h and used to stimulate HLA-A2+ PBLs. After transduction, two days after transduction, the DCs were pulsed with the MART-1 peptide for 4 h and used to stimulate HLA-A2+ PBLs. After coculture of 9 d, the cells were restimulated with the MART-1 peptide-pulsed H1.DCs and analyzed 1 wk after restimulation. To restimulate the coculture primed by autologous moDCs, the MART-1 peptide-pulsed autologous moDCs were used. The cocultures were stimulated with MART-1 peptide-pulsed H1.DCs for the third time on day 7 after the second stimulation, the cells were used as effectors to lyse HLA-A2+ and MART-1+ melanoma cells Mahne-3M 1 wk later. The HLA-A2+ and MART-1+ tumor cell line SW-480 was included for comparison.

To investigate the possible change in cytokine profile that may affect the T cell priming, we measured the concentration of several cytokines in this setting of enhanced DC and iNKT cell interaction due to CD1d upregulation. The supernatants from H1.DC and PBL cocultures were collected on day 3 and analyzed by CBA assay. Although there was some noticeable effect of baculoviral transduction on cytokine production, as shown by the reduced TNF production (Fig. 6B), we found that the BVCD1d-transduced H1.DCs induced more pronounced IFN-γ production compared with the control that used BV-transduced H1.DCs (Fig. 6B). No difference was observed in terms of IL-4 and TNF production between the experiments using BVCD1d-transduced H1.DCs and BV-transduced H1.DCs (Fig. 6B). Pretreating the CD1d-overexpressing H1.DCs with anti-CD1d Ab reduced IFN-γ production compared with treatment with isotype control (Fig. 6C). These results suggest that CD1d upregulation may induce a proinflammatory cytokine profile that favors T cell priming by DCs.

To evaluate the involvement of endogenous lipid Ag in the stimulatory effect of CD1d upregulation, the BVCD1d-transduced human DCs were treated with glycolipid biosynthesis inhibitors PPMP or NB-DGJ 1 d after baculoviral transduction. The results showed that such treatment inhibited the T cell-priming ability of CD1d-overexpressing HLDCs and moDCs (Fig. 6D), suggesting the contribution of endogenous lipid synthesis in the adjuvant effect. Furthermore, to exclude the possible phenotype difference between BV- and BVCD1d-transduced human DCs that may affect T cell priming ability, the expression levels of HLA-A2 and CD80 were measured 2 d after transduction. Similar HLA-A2 and CD80 expression levels were observed in BV- and BVCD1d-transduced human DCs (Fig. 6E), which indicates that the stimulatory effect of CD1d upregulation is not due to the upregulated expression of HLA class I or costimulatory molecules in BVCD1d-transduced DCs.

Discussion
Manipulation of human DCs to prime strong antitumor immunity will benefit patients, as demonstrated by the first U.S. Food and

FIGURE 3. CD1d upregulation in H1.DCs enhances priming of CD8+ T cells against tumor Ag. (A) The induction of tumor Ag-specific T cell response by CD1d-overexpressing H1.DCs. H1.DCs were first transduced with BV or BVCD1d and then pulsed with α-GC for 24 h, 1 d after transduction. Two days after transduction, the DCs were pulsed with the MART-1 peptide for 4 h and used to stimulate HLA-A2+ PBLs. After coculture of 9 d, the samples were stained with anti-CD3, anti-CD8, and A*0201/ELAGIGILTV Pentamer and analyzed by flow cytometer. Autologous moDCs without baculoviral transduction were pulsed with α-GC and the MART-1 peptide in the same way and included for comparison. The numbers in the representative contour plots indicate the percentage of Pentamer+ CD8+ cells in the CD3+ population. (B) Quantitative analysis of the above experiment (mean ± SD, n = 6). The statistical significance of differences was determined by the two-sided Student t test. A p value < 0.05 was considered statistically significant.

Enhancing T cell priming by CD1d upregulation depends on DC and iNKT cell interaction
To determine whether the stimulatory effect of CD1d upregulation in human DCs on Ag-specific T cell priming is CD1d-restricted, the BVCD1d-transduced, MART-1 peptide-loaded human DCs were incubated with anti-CD1d Ab before coculture with PBLs. For both CD1d-overexpressing H1.DCs and moDCs, pretreatment with anti-CD1d blocking Ab severely decreased the MART-1 peptide-specific CD8+ T cell responses compared with pretreatment with an isotype control (Fig. 6A). This suggests that the stimulatory effect of CD1d upregulation on T cell priming is CD1d-dependent. Because CD1d mediates intercellular contact between DCs and iNKT cells, this finding also indicates that the effect of CD1d upregulation on T cell priming may be associated with the interaction between DCs and iNKT cells.
Drug Administration-approved DC-based vaccine for prostate cancer (2). However, such a patient-customized vaccine is faced with inherent problems, such as limited DC number, high variability in DC quality and function, serious logistic issues, and high production costs. From an industrial standpoint, a Herculean effort is required to produce such an autologous cell therapy on a large scale (2). However, hESC-DCs may provide a potential and interesting solution to those problems, hESC-DCs are much cheaper to produce because of the feasibility for large-scale production. They can be functionally defined to ensure therapeutic efficacy via quality control of the DC product. The use of hESC-DCs has fewer logistic issues because the products may go directly from the manufacturers to clinics. Moreover, the production of hESC-DCs is scalable to provide an unlimited number of DCs, which will benefit patients who require multiple doses of vaccines.

Despite the potential of hESC-DCs, only limited studies are available on the immunological functions of this novel DC source. Typically, the hESC-DCs were loaded with viral Ags and tested for their ability to stimulate specific T cell clones (4, 5); however, their ability to present tumor Ag to induce an antitumor response needs to be studied further. Although it is possible to use hESC-DCs directly as allogeneic vaccine vehicles to induce antitumor immunity (25), better efficacy may be obtained with semiallogeneic DCs; DCs are deliberately matched to a patient’s HLA class I repertoire to present tumor Ag but not necessarily to HLA class II (26). However, to use the semiallogeneic hESC-DCs as a cancer vaccine, one potential hurdle is the fact that the allogeneic CTLs may eliminate the hESC-DCs, thereby compromising their efficacy. Hence, it is critical for the hESC-DCs to initiate a tumor Ag-specific CTL response quickly and effectively. Obviously, to induce such potent antitumor immunity, genetic manipulation of DCs is an effective strategy. Although it was shown that the genetically manipulated hESCs using PD-L1 gene are able to produce hESC-DCs with immunoregulatory function (3), the genetic manipulability of hESC-DCs themselves and the strategy to enhance the immunostimulatory function of hESC-DCs have not been demonstrated. Interestingly, a study using semiallogeneic hESC-DCs suggested that the HLA class II-mediated alloreaction may provide potent Th1-type cytokine help for the CTL response (5); however, this kind of T cell help may be elusive and highly variable among patients because of its dependence on HLA class II mismatch. To induce a potent CTL response against a weak tumor Ag, more effective and defined adjuvant help will definitely be an advantage. Therefore, we resorted to iNKT cell help to tackle such issues, because the iNKT cell population is defined and exists in all individuals. All human iNKT cells express the same iNKT TCRs that recognize CD1d-presented Ags (7). As such, manipulation of the iNKT cell help may provide a convenient strategy to enhance the hESC-DC immunogenicity. To our knowledge, we report for the first time that hESC-DCs are genetically manipulable using baculoviral vector carrying the CD1d gene, and CD1d overexpression on hESC-DCs boosts their ability to induce antitumor immunity by exploiting human iNKT cell adjuvant activity.

To use iNKT cell adjuvant activity, the potent α-GC was commonly used as stimulus (27, 28). It was demonstrated in mice that α-GC enhances malaria vaccine-induced protective immunity (29), and α-GC–loaded antigenic peptide-pulsed DCs are more efficient in priming CTLs than antigenic peptide-pulsed DCs (30). However, as indicated by limited studies using human iNKT cells, direct translation of those mouse immunological findings into the clinical setting remains difficult. For example, in two studies using human cells, suppression of Ag-specific CTL expansion was observed with the addition of free α-GC (31, 32). This suppression effect was possibly due to the Th2 cytokines released by CD4+ iNKT cells (32) or the lysis of APCs and CD1d-bearing activated T cells (31). These studies also imply that the use of free α-GC could be an issue in the clinical setting, because the free glycolipid may be presented by DCs, as well as by B cells and monocytes that may result in various iNKT cell responses and different immunological outcomes. To avoid such issues, α-GC and tumor Ag double-loaded human moDCs were used; however, α-GC loading did not improve CTL priming by moDCs, although some positive effect was shown with IL-12–overexpressing moDCs (33). Thus, these contrasting results obtained from mouse and human cell studies suggest that
mouse and human iNKT cells may respond differently to α-GC stimulation. To harness human iNKT cell adjuvant activity, a novel activation strategy, which is totally different from that established in mouse models, is required.

We reason that the use of α-GC is one possible explanation for the negative results observed in the above-mentioned human cell studies. This potent agonist may overstimulate human iNKT cells and result in elimination of the α-GC–loaded DCs (16, 17, 31, 34). Fujii et al. (35) also failed to enhance T cell priming by using α-GC– and OVA peptide-loaded DCs, and they speculated that it may have been due to the elimination of such DCs by the activated iNKT cells or specific CD8+ T cells. Therefore, overstimulation of iNKT cells could certainly pose a problem for using the α-GC and tumor Ag double-loaded DC strategy to induce antitumor immunity, indicating that optimal stimulation of iNKT cells could be critical. The strength of such optimal stimulation may lie between that provided by the physiological level of endogenous iNKT ligands and that rendered by the potent agonist like α-GC. Hence, upregulation of Ag/CD1d complexes on the DC surface may provide such optimal strength to activate iNKT cells. To this end, we propose a novel strategy that uses enforced overexpression of CD1d on human DCs to employ the human iNKT cell adjuvant activity. We demonstrated that such CD1d-overexpressing human DCs were effective in promoting the tumor Ag-specific CTL response.

Several factors may facilitate T cell priming by the CD1d-overexpressing human DCs. First, the direct interaction between CD1d-overexpressing DCs and iNKT cells is crucial. As shown in the murine system, the ligation of CD40L on iNKT cells to CD40 on DCs may trigger DC maturation and induce strong adaptive immunity (27), although it is unclear whether such a mechanism also exists in the human system. In our study, pretreatment with anti-CD1d blocking Ab severely reduced the T cell priming ability of CD1d-overexpressing human DCs, suggesting the importance of such DC and iNKT cell interactions in this context. It is possible that, through this direct interaction, iNKT cells license the DCs to appropriately activate CD8+ T cells. However, the signaling pathways involved remain to be elaborated. Second, the favorable cytokine profile for T cell priming is induced by CD1d-overexpressing DCs. It is known that the cytokine environment plays an important role at the initiation stage of an adaptive immune response, and the proper activation of iNKT cells may provide the necessary cytokines to jump-start such adaptive immunity (13). As shown in the current study, the CD1d-overexpressing hESC-DCs induced a proinflammatory cytokine profile that may favor the initiation of...
an Ag-specific T cell response. Third, baculoviral transduction may provide a positive stimulatory effect in the CD1d-overexpressing DCs. It was shown that baculovirus, as a dsDNA virus, may act on mouse myeloid DCs via a TLR-independent pathway (36) and may induce functional maturation in human moDCs (37). In our study, although baculoviral transduction alone did not enhance the T cell priming ability of hESC-DCs or moDCs, its effect cannot be excluded. Interestingly, we also found that α-GC loading did not increase, but instead decreased, the immunostimulatory function of CD1d-overexpressing hESC-DCs, which may further underscore the importance of proper iNKT cell activation in initiating the CTL response.

Overall, this study demonstrated that the enforced expression of CD1d on hESC-DCs enhanced the DC efficacy in priming CD8+ T cells against tumor Ag. The ability to generate large amounts of uniform hESC-DCs, their genetic manipulability, and their competence in inducing antitumor immunity indicate that hESC-DCs can potentially be used as an unlimited cell source to produce off-the-shelf DC-based vaccines.

Acknowledgments
We thank other laboratory members for helpful discussion and support.

Disclosures
The authors have no financial conflicts of interest.

References
SUPPLEMENTAL INFORMATION

Enhancing Immunostimulatory Function of Human Embryonic Stem Cell-derived Dendritic Cells by CD1d-overexpression

Jieming Zeng*, Mohammad Shahbazi*†, Chunxiao Wu* and Shu Wang*,†

*Institute of Bioengineering and Nanotechnology, Singapore
†Department of Biological Sciences, National University of Singapore, Singapore
Supplemental Figure 1. Generation of human DCs from hESCs using a three-step culture protocol. (A-D) Morphological changes of the HES-1 during differentiation process. (A) Differentiated HES-1 cells grown on OP9 cells for 9 days. (B) Harvested HES-1/OP9 co-culture grown in suspension with GM-CSF for 10 days. (C) HES-1/OP9 co-culture further differentiated in suspension with GM-CSF and IL-4 for 8 days. (D) Morphology of the HES-1-derived DCs cultured on normal cell culture dish. (E-G) Morphology of the differentiated H1 before and after purification. To purify H1.DCs from the differentiated H1, the pre-sort population (E) was stained with anti-CD209 antibody and sorted by flow cytometer to generate a homogenous post-sort population (F). The post-sort H1.DCs grew as attached cells on normal cell culture surface (G).
Supplemental Figure 2. Phenotypes of DCs differentiated from H1, H9 and HES-1 hESCs. DCs were generated with the three-step culture method and analyzed by flow cytometry. Histograms show the staining by antibodies against indicated markers (red line) and their isotype controls (black line). The percentages of the positive cells are indicated.
Supplemental Figure 3. In vitro expansion of iNKT cells induced by CD1d-overexpressing H1.DCs. The H1.DCs were pulsed with α-GC one day after baculoviral transduction for 24 hours and co-cultured with PBLs or pan-T cells two days after baculoviral transduction. Free α-GC was added in co-culture and iNKT cells were then analyzed by flow cytometry 7 days after. Dot plots and the percentages of the iNKT cells in total T cells are shown. (A) iNKT cell expansion in co-cultures using human PBLs or pan-T cells as responders. (B) The comparison of iNKT cell expansion in the presence and absence of α-GC using PBLs as responders. (C) The comparison of iNKT cell expansion induced by autologous moDCs and CD1d-overexpressing H1.DCs using PBLs as responders.