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Hybrids of Dendritic Cells and Tumor Cells Generated by Electrofusion Simultaneously Present Immunodominant Epitopes from Multiple Human Tumor-Associated Antigens in the Context of MHC Class I and Class II Molecules¹

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Abstract

Hybrid cells generated by fusing dendritic cells with tumor cells (DC-TC) are currently being evaluated as cancer vaccines in preclinical models and human immunization trials. In this study, we evaluated the production of human DC-TC hybrids using an electrofusion protocol previously defined for murine cells. Human DCs were electrically fused with allogeneic melanoma cells (888mel) and were subsequently analyzed for coexpression of unique DC and TC markers using FACS and fluorescence microscopy. Dually fluorescent cells were clearly observed using both techniques after staining with Abs against distinct surface molecules suggesting that true cell fusion had occurred. We also evaluated the ability of human DC-TC hybrids to present tumor-associated epitopes in the context of both MHC class I and class II molecules. Allogeneic DCs expressing HLA-A*0201, HLA-DR β 1*0401, and HLA-DR β 1*0701 were fused with 888mel cells that do not express any of these MHC molecules, but do express multiple melanoma-associated Ags. DC-888mel hybrids efficiently presented HLA-A*0201-restricted epitopes from the melanoma Ags MART-1, gp100, tyrosinase, and tyrosinase-related protein 2 as evaluated by specific cytokine secretion from six distinct CTL lines. In contrast, DCs could not cross-present MHC class I-restricted epitopes after exogenously loading with gp100 protein. DC-888mel hybrids also presented HLA-DR β 1*0401- and HLA-DR β 1*0701-restricted peptides from gp100 to CD4⁺ T cell populations. Therefore, fusions of DCs and tumor cells express both MHC class I- and class II-restricted tumor-associated epitopes and may be useful for the induction of tumor-reactive CD8⁺ and CD4⁺ T cells in vitro and in human vaccination trials.

Dendritic cells (DCs)³ are potent APCs that are capable of stimulating both naive CD4⁺ Th cells and CD8⁺ CTLs (1). Therefore, DCs are being extensively evaluated as vehicles for Ag delivery in immunotherapies for the treatment of patients with cancer. Many techniques have been used to load DCs with tumor-associated Ags, including pulsing with synthetic peptides that represent T cell epitopes (2,3). This strategy has been used in several human clinical vaccination trials (4,5); however, it is limited to patients who express the particular peptide

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³Abbreviations used in this paper: DC, dendritic cell; DC-TC, DC fused with tumor cell; PEG, polyethylene glycol; TRP, tyrosinase-related protein; VSV, vesicular stomatitis virus; GFP, green fluorescent protein; CIITA, class II transactivator; TIL, tumor-infiltrating lymphocyte.

MHC-restricting molecule. Alternatively, DCs have been pulsed with recombinant proteins (6) or transduced with recombinant viruses (7-12). These approaches circumvent the MHC restrictions associated with peptides but are generally limited to individual proteins. Because many tumors display heterogenous expression of target Ags (13), strategies that induce T cell responses against multiple proteins may be more efficacious. Another concern is that some of these Ag loading techniques facilitate the presentation of immunogenic viral or bacterial epitopes in addition to those from the tumor-associated protein.

Other Ag presentation systems have been developed to stimulate polyclonal immune responses against multiple tumor-associated proteins, including those that are known, undefined, shared, or unique. For example, DCs have been loaded with killed tumor cells (14) and tumor lysates (15,16). These approaches may be inefficient for stimulating polyclonal CTL responses because some proteins are not efficiently cross-presented even by DCs that have mechanisms which allow exogenously loaded proteins to enter MHC class I-processing pathways (1). Alternatively, DCs have been pulsed with peptides eluted from tumor cells (17) and have been transfected with tumor-derived RNA (18,19). In murine models, these approaches have induced protective and therapeutic immune responses against tumors and are currently being evaluated in human clinical vaccination trials.

Another evolving strategy for inducing immunotherapeutic responses against cancer is the use of hybrid cells generated by fusing DCs with tumor cells (DC-TC) (20-22). Theoretically, DC-TC hybrids possess properties of both parental cell types necessary for inducing primary CD4⁺ and CD8⁺ T cell responses. Namely, fusions of DCs and tumor cells should express shared and unique tumor-associated Ags, high levels of MHC class I and class II molecules, and adhesion and costimulatory molecules. Vaccinations with DC-TC hybrids have stimulated protective and therapeutic immune responses in several rodent tumor models including carcinomas, lymphomas, melanomas, and gliomas (23-29). In addition, several in vitro studies with human cells have suggested that DC-TC hybrids can present relevant tumor-associated Ags in the context of class I HLA molecules and induce tumor-reactive CTL (30-33). However, in some of these studies, the production of multinucleated hybrids with fused cell membranes was not clearly documented. Because previous investigations have demonstrated that physical mixtures of DCs and tumor cells can sometimes induce effective CTL responses (34), some results pertaining to the enhanced immunogenicity of DC-TC hybrids have been difficult to interpret.

Two human clinical trials have been reported in which patients with melanoma (35) or malignant glioma (36) were vaccinated with autologous DC-TC hybrids made with the fusogenic agent polyethylene glycol (PEG). Although the treatments were safe and well-tolerated, few clinical responses were observed. An alternate technique for generating DC-TC hybrids is to expose cells to electric fields (37). A cell mixture is first treated with an alternating nonuniform electric current of low strength that causes cells to align, forming tight membrane contacts. Subsequently, a direct current pulse with high intensity but short duration disrupts cell membranes reversibly. After cessation of this pulse, membrane resealing occurs between cells in close contact. Results from several investigations have demonstrated that electrofusion can generate genuine DC-TC hybrids (22,28,33). In FACS profiles and confocal micrographs, fused cells appear as a distinct population that are dually fluorescent for individual cell-specific markers and are clearly distinguishable from DCs or tumor cells alone that remain in the mixture. Furthermore, hybrids can be seen as individual multinucleated cells on Giemza-stained cytocentrifuge slides.

Vaccinations with DC-TC hybrids generated by electrofusion have been shown to be therapeutic in murine tumor models (28). Namely, intranodal injection of DC-TC fusion cells with IL-12 as an adjuvant eradicated established s.c. GL261 gliomas and D5LacZ3 pulmonary

metastases. To adapt this technology to human clinical trials, we evaluated the ability of electrofused human DC-TC hybrids to present tumor-associated Ags in the context of both MHC class I and class II molecules. Allogeneic DC-TC hybrids were produced by fusing DCs expressing HLA-A*0201, -DR β 1*0401, and -DR β 1*0701 with melanoma cells (888mel) that express none of these HLA molecules. The generation of membrane-fused DC-888mel hybrids was verified using a combination of FACS and fluorescence microscopy. Based on specific IFN- γ secretion by six unique tumor-reactive CTL lines, DC-888mel hybrids presented immunodominant epitopes from the melanoma Ags, MART-1, gp100, tyrosinase, and an alternate isoform of tyrosinase-related protein (TRP) 2 (TRP2-6b) in the context of HLA-A*0201. Furthermore, these hybrids were specifically recognized by CD4⁺ T cell lines reactive with gp100 epitopes in the context of HLA-DR β 1*0401 and -DR β 1*0701. These results suggest that electrofused human DC-TC hybrids may elicit both CD4⁺ and CD8⁺ T cell responses, and hence, may be useful in human cancer vaccine trials. They may also be useful for the identification of new human tumor-associated Ags, particularly for cancers for which it has been difficult to establish tumor-reactive T cell populations by traditional means. In addition, human DC-TC hybrids may enable the ex vivo generation of tumor-reactive T lymphocytes for use in adoptive therapy protocols.

Materials and Methods

Cell lines and reagents

Human melanoma cell lines and EBV-transformed B cells were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Human lymphocytes were cultured in complete medium consisting of RPMI 1640, 2 mM L-glutamine, 50 U/ml penicillin, 50 μ g/ml streptomycin (Invitrogen) and 10% heat-inactivated human AB serum (Gemini Bio-Products, Woodland, CA; Valley Biomedical, Winchester, VA). Multiple melanoma-reactive T cell lines and clones (38-45) were used to evaluate the presentation of MHC-restricted epitopes by DC-TC hybrids as presented in Table I.

Expression of MART-1 and gp100 in melanoma cell lines was assessed by FACS using mAbs (M2-7 C10 from Dr. Y. Kawakami (Keio University School of Medicine, Tokyo, Japan; Ref. 46; HMB45 from Enzo Diagnostics (Farmingdale, NY)), and the presence of tyrosinase was assessed by immunohistochemistry (13). Expression of TRP2-6b and NY-ESO-1 was previously evaluated on the basis of specific IFN- γ secretion by two T cell clones, MB4 and M8, respectively, that specifically recognize peptides from these proteins in the context of HLA-A*0201 (41). Although the 888 melanoma cell line (888mel) did not express HLA-A*0201, recognition of this cell line, and hence, expression of TRP2-6b and NY-ESO-1, was evaluated after stable transfection with cDNA encoding HLA-A*0201. The F002R melanoma cell line (F002Rmel) did not naturally express gp100, but it had previously been transduced with a vesicular stomatitis virus (VSV)-pseudotyped retroviral vector encoding either gp100 or green fluorescence protein (GFP) (44). Expression of HLA class II molecules on the surfaces of melanoma cells was up-regulated by transduction with a retroviral construct encoding the HLA class II transactivator (CIITA) gene as previously described (47). The expression of cell surface HLA class I and class II molecules on melanoma cells was confirmed by FACS using mAbs against HLA-A2 (One Lambda, Conestoga, CA), HLA-DR (L243; BD Biosciences, Franklin Lakes, NJ), HLA-DR4 (Accurate Chemical and Scientific, Westbury, NY), and HLA-DR7 (Pel-Freez Biologicals, Rogers, AR). In addition, HLA haplotypes of cell lines were determined by DNA sequencing in the HLA Laboratory (National Institutes of Health). The expression of MART-1, gp100, tyrosinase, TRP2-6b, NY-ESO-1, HLA-A*0201, HLA-DR β 1*0401, and HLA-DR β 1*0701 in melanoma cell lines was as follows: 888mel (MART-1⁺, gp100⁺, tyrosinase⁺, TRP2-6b weak, NY-ESO-1⁻, HLA-A*0201⁻, HLA-

DR β 1*0401⁻, HLA-DR β 1*0701⁻), 624mel CIITA (MART-1⁺, gp100⁺, tyrosinase⁺, TRP2-6b⁺, NY-ESO-1⁺, HLA-A*0201⁺, HLA-DR β 1*0401⁺, HLA-DR β 1*0701⁺), 526mel CIITA (MART-1⁺, gp100⁺, tyrosinase⁺, TRP2-6b⁺, NY-ESO-1⁻, HLA-A*0201⁺, HLA-DR β 1*0401⁺, HLA-DR β 1*0701⁻), F002Rmel CIITA-gp100 (gp100⁺, HLA-A*0201⁺, HLA-DR β 1*0401⁻, HLA-DR β 1*0701⁺), F002Rmel CIITA-GFP (gp100⁻, HLA-A*0201⁺, HLA-DR β 1*0401⁻, HLA-DR β 1*0701⁺), 1088mel CIITA (gp100⁺, HLA-DR β 1*0401⁺, HLA-DR β 1*0701⁻), 1978mel CIITA (gp100⁺, HLA-DR β 1*0401⁻, HLA-DR β 1*0701⁺), and 1861mel CIITA (gp100⁺, HLA-DR β 1*0401⁺, HLA-DR β 1*0701⁻).

Electrofusion of DCs and tumor cells

Immature DCs were prepared as previously described (48). Briefly, adherent PBMC were cultured in RPMI 1640 with 10% heat-inactivated FBS containing 2000 IU/ml GM-CSF (Amgen-ImmuneX, Seattle, WA) and 1000 IU/ml IL-4 (R&D Systems, Minneapolis, MN). Five days later, DC maturation was induced with 10 ng/ml TNF- α (Sigma-Aldrich, St. Louis, MO) and 1 μ g/ml PGE₂ (Sigma-Aldrich). On day 7, electrofusion of the DCs and 888mel cells was performed as previously described with slight modification (28). In most experiments, 888mel cells were labeled with CFSE (Molecular Probes, Eugene, OR) before electrofusion: 10⁷ cells/ml in HBSS were labeled in the presence of 5 μ M dye for 10 min at 37°C, and labeling was terminated by dilution with ice-cold HBSS. In all experiments, 888mel cells were irradiated with 10,000 cGy before electrofusion. To induce fusion, DCs and irradiated tumor cells were mixed at a ratio of 1:1 and were suspended in a 5% glucose solution containing 0.1 mM Ca(CH₃COO)₂, 0.5 mM Mg(CH₃COO)₂, and 0.3% BSA. The pH of the fusion medium was adjusted to 7.2–7.4 with L-histidine (all chemicals from Sigma-Aldrich). After centrifugation, the cells were resuspended in the same medium in the absence of BSA. Routinely, 1 ml of cell suspension containing 1 \times 10⁷ cells were processed using a specially designed concentric fusion chamber. For electrofusion, a pulse generator (model ECM 2001; BTX Instruments, Genetronics, San Diego, CA) was used for application of the field pulses. Cell alignment was first induced by dielectrophoresis with an alternating current (ac) pulse of 150 V/cm for 10 s. Subsequently, cell fusion was triggered by application of a single square wave direct current (dc) pulse of 1200 V/cm for 25 μ s. The fusion mixture was allowed to rest for 5 min before suspension in RPMI 1640 with 10% heat-inactivated FBS. After overnight incubation at 37°C, cells were separated on the basis of adherence. The nonadherent population was aspirated and consisted primarily of residual DCs. The adherent population was harvested using a solution of 0.05% trypsin and 0.02% EDTA (Trypsinversene; BioWhittaker, Walkersville, MD) and contained predominantly fusion hybrids and tumor cells.

FACS analyses and fluorescence microscopy

In some experiments, 888mel cells were labeled with CFSE before electrofusion to enable visualization with FACS or fluorescence microscopy. After fusion, DC-888mel hybrids were detected by staining with PE-conjugated mAbs against DC cell surface markers including CD11c, CD40, CD80, CD86, CD83, and HLA-DR (BD PharMingen, San Diego, CA). Alternatively, in other experiments, 888mel cells were not labeled before fusion, and DC-TC hybrids were detected by costaining with mAbs against markers expressed on either cell type. Namely, FITC-conjugated anti-HLA-DR was used as a DC marker concomitantly with mAbs against gp100 (Enzo Diagnostics) or HLA-A24 (One Lambda), both of which were only expressed in the 888mel cells. These Abs were visualized using a secondary PE-conjugated goat anti-mouse IgG (Caltag Laboratories, Burlingame, CA).

Cytokine release assays

Recognition of target cells by melanoma-reactive CD8⁺ and CD4⁺ T cell lines and clones was evaluated on the basis of specific IFN- γ secretion. In one set of experiments, cytokine secretion

was measured in response to immature DCs loaded with recombinant gp100 protein or melanoma cell lysates, as well as DCs transduced with an adenoviral vector encoding gp100. Recombinant proteins were commercially produced and purified (Novavax, Columbia, MD). DCs expressing HLA-A*0201 and HLA-DR β 1*0701 were incubated with 10 μ g/ml gp100 protein (or NY-ESO-1 as a negative control), overnight at 37°C in 96-well plates (1×10^5 cells/well; 100 μ l/well). Alternatively, lysates of F002Rmel CIITA-gp100 cells (or F002Rmel CIITA-GFP as a negative control) were prepared by exposing the cells to five freeze-thaw cycles. Lysates were incubated with DCs at a ratio of one DC to one melanoma cell equivalent overnight at 37°C in 96-well plates (1×10^5 cells/well; 100 μ l/well). DCs were also transduced with recombinant adenoviruses encoding gp100 (or MART-1 as a negative control) (Genzyme, Cambridge, MA) as previously described (10) and were incubated 24–48 h at 37°C in 96-well plates (1×10^5 cells/well; 100 μ l/well). As positive controls for T cell function, specific IFN- γ secretion was measured in response to peptide-loaded DCs and melanomas. For HLA-A*0201-restricted CD8 $^+$ T cell populations, DCs were incubated with 1 μ g/ml of the appropriate peptide 1–3 h at 37°C. For HLA class II-restricted CD4 $^+$ T cells, DCs were incubated with 50 μ g/ml of the appropriate peptide ~3 h at 37°C. In addition, the melanoma cell lines, F002Rmel CIITA-gp100 and -GFP, were harvested. Responder T cells (10^5) were coincubated with 10^5 stimulator cells (250 μ l total) ~20 h at 37°C, and the concentration of human IFN- γ in coculture supernatants was measured by ELISA (Pierce-Endogen, Cambridge, MA).

Recognition of DC-888mel hybrid cells by melanoma-reactive CD8 $^+$ and CD4 $^+$ T cell lines and clones was also evaluated on the basis of specific IFN- γ secretion. Responder T cells (10^5) were coincubated with $1-4 \times 10^4$ fusion cells in 96-well plates ~20 h at 37°C, and the concentration of human IFN- γ in coculture supernatants was measured by ELISA (Pierce-Endogen). As positive controls for T cell function, specific IFN- γ secretion was measured in response to peptide-loaded target cells and melanomas. For HLA-A*0201-restricted CD8 $^+$ T cell populations, T2 cells were incubated with 1 μ M of the appropriate peptide 1–3 h at 37°C. For class II HLA-restricted CD4 $^+$ T cells, EBV-transformed B cells expressing HLA-DR β 1*0401 or HLA-DR β 1*0701 were incubated with 50 μ M of the appropriate peptide ~3 h at 37°C. In addition, melanoma cell lines expressing various combinations of HLA-A*0201, HLA-DR β 1*0401, and HLA-DR β 1*0701, were harvested. Responder T cells (10^5) were coincubated with 10^5 stimulator cells, and the concentration of human IFN- γ in coculture supernatants was measured by ELISA (Pierce-Endogen).

Results

Characterization of human DC-melanoma cell hybrids

Phenotypic analysis of human DCs and hybrids of DCs fused with 888 melanoma cells (DC-888mel) was performed by FACS. Before electrofusion, DCs displayed markers consistent with those of mature DCs (Fig. 1); they expressed CD11c but not the monocyte/macrophage marker CD14 or the NK cell marker CD57. In addition, they expressed HLA class I and class II molecules, costimulatory molecules B7.1 and B7.2, and maturation markers CD40 and CD83. In >50 experiments in which entire viable cell populations were gated, >90% of cells simultaneously expressed these DC markers while <4% expressed the T cell marker CD3, and <1% expressed the B cell marker CD19 or the monocyte/macrophage marker CD14.

Electrofusion of DCs and 888mel cells generated heterokaryons that expressed both green fluorescence (CFSE) from the tumor cells and several DC markers (Fig. 2). After electrofusion, cells were incubated overnight at 37°C and were subsequently separated on the basis of adherence. The majority of dually fluorescent fusion cells were detected in the adherent cell population. Also contained in the adherent fraction was a significant population of melanoma cells that were only positive for CFSE. As estimated by FACS analyses characteristic of those

presented in Fig. 2, the percentages of DC-TC fusion cells in adherent populations ranged from 12 to 52%. In contrast, the nonadherent population primarily contained DCs, with smaller populations of hybrid cells and melanoma cells.

FACS analyses are useful for estimating fusion efficiencies, but they may be misleading because dually fluorescent events could represent cell aggregates or DCs that have engulfed tumor cell debris as opposed to membrane-fused hybrids. To evaluate whether true cell fusions were produced in the experiments presented in this study, fluorescence microscopy was used to evaluate the quality of dual fluorescence on individual cells. CFSE-labeled 888mel cells were fused with DCs, and the adherent population was stained with PE-conjugated anti-HLA-DR as a DC marker (Fig. 3). As a negative control, adherent 888mel cells that had undergone the electrofusion process were also stained with PE-conjugated anti-HLA-DR. In the DC-888mel fusion population, individual cells were clearly positive for both CFSE and HLA-DR. Also, some cells were observed that were only positive for green fluorescence, as expected from the FACS analyses. However, in the preparation consisting only of tumor cells, no HLA-DR expression was observed. Close examination of these cytopsin slides also revealed that dually positive fusion cells contained multiple nuclei further suggesting that true cell fusion had occurred.

Although the fluorescence micrographs suggested that cell fusion had, in fact, occurred, the argument could still be made that dually fluorescent cells represent DCs that had engulfed tumor cell debris because CFSE is an intracellular stain. Therefore, to evaluate whether fusion of cell membranes had occurred, we costained DC-888mel fusion cells with Abs against two distinct surface markers (Fig. 4). In these experiments, 888mel cells were not prelabeled with CFSE. 888mel cells expressed HLA-A24 on their surfaces whereas DCs from donor 7 did not. After electrofusion of either DCs and unlabeled 888mel cells or of 888mel cells alone, the adherent populations were stained with anti-HLA-A24 that was indirectly coupled to PE through a labeled secondary Ab. Subsequently, the cells were counterstained with FITC-conjugated anti-HLA-DR. In the DC-888mel fusion population, individual cells were clearly positive for both HLA-A24 and HLA-DR. Also, some cells were observed that only expressed individual cell markers. However, only HLA-A24⁺ cells were found in the tumor cell alone preparation.

Lack of cross-presentation of gp100 by DCs

DCs are potent APCs that are sometimes able to cross-present MHC class I-restricted epitopes from exogenously loaded Ags (1,6). To determine whether gp100 protein could be cross-presented by DCs, IFN- γ secretion by two CTL lines (CK3H6 and tumor-infiltrating lymphocyte (TIL) 1200) that recognize gp100 epitopes in the context of HLA-A*0201 was measured in response to three different HLA-A*0201⁺ DCs loaded with recombinant gp100 protein or lysates of melanoma cells expressing gp100 after retroviral transduction (F002Rmel CIITA + VSV-gp100) (Table II). DCs loaded with recombinant NY-ESO-1 protein and lysates of gp100⁻ melanoma cells expressing GFP after retroviral transduction (F002Rmel CIITA + VSV-GFP) were included as negative controls. In addition, whole F002Rmel CIITA cells that naturally expressed HLA-A*0201 were included as controls for T cell function after transduction with VSV-gp1000 or VSV-GFP. Furthermore, DCs were pulsed with relevant peptides (gp100:209-217 for CK3H6 and gp100:154-162 for TIL 1200) and were transduced with an adenoviral vector encoding gp100 as positive control for Ag presentation by the DCs in the context of HLA-A*0201. CK3H6 specifically recognized each of the three DC populations after pulsing with gp100:209-217 and after transduction with adenovirus encoding gp100. However, no specific IFN- γ secretion was observed in response to DCs loaded with whole gp100 protein or melanoma cell lysates. The same trend was observed for TIL 1200 that

specifically recognized gp100:154-162; however, the total amounts of IFN- γ secreted were significantly lower.

We also evaluated MHC class II-restricted Ag presentation by these same DC populations. IFN- γ secretion was measured by a CD4⁺ T cell clone (B104) that specifically recognized gp100:170-190 in the context of HLA-DR β 1*0701, which was concomitantly expressed on all three DCs. B104 specifically recognized DCs that had been pulsed with gp100:170-190 and those that had been adenovirally transduced with gp100. In addition, B104 specifically released IFN- γ in response to DCs loaded with recombinant gp100 protein and lysates of melanoma cells expressing gp100. These results demonstrated the integrity of the gp100 protein and lysate preparations in that DCs were capable of processing exogenously loaded Ags and presenting relevant epitopes in the context of MHC class II molecules.

Presentation of MHC class I- and class II-restricted epitopes by DC-888mel hybrids

To determine whether DC-TC hybrids presented relevant MHC class I-restricted tumor-associated epitopes, IFN- γ secretion by multiple HLA-A*0201-restricted, melanoma-reactive CTLs was measured in response to allogeneic DC-888mel hybrids consisting of HLA-A*0201⁺ DCs and HLA-A*0201⁻ 888mel cells (Table III). Three gp100-reactive CTL lines, each of which recognized a unique peptide, specifically released IFN- γ in response to DC-888mel fusions prepared from two different donors. These T cells did not recognize a physical mixture of DCs and irradiated 888mel cells, nor did they secrete IFN- γ in response to 888mel cells that had undergone the electrofusion procedure alone. Furthermore, in a separate experiment, these T cells were not stimulated by fusions of HLA-A*0201⁻ DCs and 888mel cells. Similar results were also observed using HLA-A*0201-restricted CTLs that specifically recognized epitopes from MART-1, tyrosinase, and TRP2-6b. The amounts of IFN- γ secreted by several of these CTLs in response to cells from the adherent fraction of the DC-888mel fusion preparations were comparable to that released in response to HLA-A*0201⁺ melanoma cells. This suggested that DC-888mel hybrids were potent stimulators because significantly fewer cells were used in these samples compared with melanoma cells. For example, in the first experiment described in Table III, 3.75×10^4 total fusion cells were used as targets as opposed to 10^5 tumor cells. However, only 29% of the adherent DC-888mel fusion population from donor 4 were hybrid cells as estimated by FACS analysis. Because heterokaryons were not separated from tumor cells or DCs, 10^4 DC-888mel hybrids from donor 4 stimulated secretion of comparable amounts of IFN- γ by several CTL lines as 10^5 melanoma cells. Two CTL populations, CK3H6 and GA3D, also secreted cytokine in response to the nonadherent fraction of the DC-888mel fusion samples. This was probably due to the small numbers of hybrids present in these fractions as observed by FACS (Fig. 2). As an additional negative control, an HLA-A*0201-restricted, NY-ESO-1-reactive CTL line was included because 888mel cells do not efficiently express this protein. These T cells did not recognize HLA-A*0201⁺ DC-888mel hybrids.

Presentation of MHC class II-restricted tumor-associated epitopes by DC-888mel hybrids was also evaluated. IFN- γ secretion by a gp100-reactive HLA-DR β 1*0401-restricted CD4⁺ T cell line and by an HLA-DR β 1*0701-restricted T cell clone was measured in response to allogeneic DC-888mel hybrids consisting of DCs expressing both of these MHC class II molecules and 888mel cells that express neither (Table IV). Both of these T cell populations specifically released cytokine in response to DC-888mel fusions prepared from two different donors. These T cells did not recognize a physical mixture of DCs and irradiated 888mel cells, nor did they secrete IFN- γ in response to 888mel cells that had undergone the electrofusion procedure alone. Furthermore, in a separate experiment, these T cells were not stimulated by fusions of 888mel cells with DCs that did not express HLA-DR β 1*0401 or -DR β 1*0701.

Discussion

The absence of an effective T cell-mediated immune response to cancer in many patients may be the result of inefficient Ag processing and presentation by tumor cells (49,50). Down-regulation of MHC molecules as well as defective Ag processing has been observed in neoplastic cells (51,52). In addition, tumor cells do not generally express costimulatory and/or adhesion molecules that may be necessary to mount a primary immune response (53). To overcome this unresponsiveness, a variety of vaccination strategies have been developed to stimulate T lymphocytes that recognize Ags expressed in tumor cells. Sporadic clinical responses have been observed in trials in which Ags or peptide epitopes have been used directly to immunize patients (54). However, these approaches have largely been unsuccessful especially when the target Ags are nonmutated self proteins (55). Therefore, the use of DCs as an adjuvant in cancer vaccine trials is currently being intensively studied because DCs are potent APCs capable of initiating primary immune responses.

Many methods have been developed for loading DCs with Ags including transfection with tumor-derived RNA or DNA (18,19), transduction with recombinant viruses (7-12), and loading with killed tumor cells (14), tumor lysates (15,16), recombinant protein (6), or synthetic peptides (3,55). Alternatively, hybrids of DCs and tumor cells have been produced using PEG or electrofusion (20-22). Theoretically, DC-TC hybrids are appealing because they should induce a polyclonal immune response, including both CD4⁺ Th cells and CD8⁺ CTL, against a multitude of shared and unique tumor-associated Ags. In preclinical murine models, DC-TC hybrids have stimulated potent protective and therapeutic immune responses to carcinomas, lymphomas, melanomas, and gliomas (23-29). However, in two recent human clinical trials, fusions of DCs and autologous tumor cells were primarily ineffective for the treatment of patients with melanoma and glioma (35,36). In both of these protocols, PEG was used as the fusogenic reagent. In one previous investigation, electrofusion was preferred over PEG-mediated fusion specifically because fusion efficiency was very low with PEG, ranging from only 0.5 to 4.5% (33). In another investigation, a direct comparison was made between DC-TC fusions made using PEG and electrofusion in a prophylactic DA3 mammary carcinoma model (25). In that study, DCs were labeled with 5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine (CMTMR) before fusion and tumor cells were prelabeled with CFSE such that fusion efficiency was monitored by FACS for dual fluorescent events. The conclusion was drawn that no significant differences were observed in either the quality of the fusion cells or in the ability of those cells to mediate protective immunotherapy. However, the FACS profile for PEG-fused cells appeared nearly identical to that for DCs and tumor cells that had simply been cocultured. Namely, both profiles indicated that these cell populations predominantly contained two separate cell types, one green and one red, with very few dual fluorescent cells. Alternatively, cells generated by electrofusion appeared to contain a much more obvious population of dual fluorescent cells. Furthermore, we have previously attempted to generate DC-TC hybrids using PEG but could not demonstrate effective fusion, partly because Abs against DC markers bound nonspecifically to tumor cells alone after PEG treatment (data not shown). Therefore, we have focused our analysis on DC-TC hybrids generated by electrofusion and have verified the occurrence of chimeric hybrids using a series of stringent criteria.

Prelabeling of DCs and tumor cells coupled with FACS analysis is a convenient means of assessing fusion efficiency. However, FACS data can be misleading because dual fluorescent events may represent cell aggregates or DCs that have engulfed tumor cell debris. Results from some previous investigations have been difficult to interpret because it was unclear whether immune responses were mediated by true membrane-fused cells or by DCs presenting Ag through uptake of tumor-associated material. In fact, several investigations have demonstrated that physical mixtures of DCs and tumor cells can elicit potent immune responses (34). To evaluate whether true fusion was achieved in the current study, we used a combination of FACS

analysis and fluorescence microscopy. In the adherent fraction of the fusion population, there was clearly a distinct population of cells that were positive for CFSE from the prelabeled melanoma cells and for several DC markers by both FACS (Fig. 2) and microscopic analyses (Fig. 3). Fluorescence micrographs also revealed that dually colored cells were multinucleated because CFSE is a cytoplasmic dye that does not stain cell nuclei. However, the argument could still be made that these cells were DCs that had engulfed tumor cell debris. Therefore, we evaluated expression of two distinct cell surface markers, namely HLA-A24 that was only expressed on 888mel and HLA-DR that was only present on the DCs before fusion. In the adherent fraction of the fusion population, there were cells that expressed both of these markers suggesting that we had, in fact, generated membrane-fused hybrids (Fig. 4).

The primary theoretical advantages of DC-TC hybrids over DCs loaded with Ags by other means are 2-fold: 1) they should be able to present epitopes from multiple tumor-associated Ags simultaneously, and 2) they should be able to present relevant epitopes in the context of both MHC class I and class II molecules. DCs have unique Ag processing pathways that enable exogenously loaded proteins to be presented in the context of MHC class I molecules (1). However, cross-presentation is, generally, not very efficient in the absence of carrier proteins or particles (6). As an example, we have never observed cross-presentation of the melanoma Ag gp100, either in the form of a recombinant protein or tumor cell lysate (Table II). In contrast, hybrids of HLA-A*0201⁺ DCs and HLA-A*0201⁻ gp100⁺ melanoma cells were clearly recognized by three CTL lines, each of which was reactive with a unique HLA-A*0201-restricted gp100 epitope (Table III). In addition, DC-888mel hybrids presented MHC class I-restricted epitopes from other melanoma Ags including MART-1, tyrosinase, and TRP2-6b. Furthermore, DC-888mel hybrids presented class II MHC-restricted epitopes from gp100. These results suggest that DC-TC hybrids may be able to induce both CTL and Th cell responses against a variety of different tumor-associated Ags.

Several in vitro and in vivo applications can be envisioned for the use of electrofused DC-TC hybrids as APCs. The immuno-therapeutic potential of these hybrid cells can be evaluated in human clinical vaccination trials. In addition, they may be useful for the identification of new tumor-associated Ags. If DC-TC hybrids can stimulate tumor-reactive CD8⁺ and CD4⁺ T lymphocytes, the antigenic specificities of those T cells could be ascertained by-screening tumor-derived cDNA libraries (56,57) or peptides eluted from tumor cells for recognition (58,59). Furthermore, fusions of DCs and tumor cells may be useful in vitro for generating populations of tumor-reactive T cells for use in adoptive therapy protocols. In several investigations, significant objective clinical response rates have been achieved by the adoptive transfer of autologous tumor-reactive T cells (60). For patients with melanoma, it has been comparatively easy to raise these T cell populations from TIL. In addition, melanoma cells seem to be uniquely immunogenic in that they are often directly able to stimulate tumor-reactive CTL in vitro without manipulation such as the introduction of costimulatory molecules or cytokine adjuvants. In contrast, it has been significantly more difficult to raise such T cell populations for other cancers. Even in the context of renal cell carcinoma, which is often responsive to immunotherapy with IL-2 (61), it has been difficult to raise tumor-reactive T cells in vitro, either from TIL or from PBMC stimulated with whole tumor cells. In addition, mounting evidence suggests that CD4⁺ T lymphocytes may play a critical role in the induction and persistence of tumor-reactive CD8⁺ T cells (60,62-64). Therefore, the use of DC-TC hybrids as APCs may be a viable means for raising heterogeneous populations of tumor-reactive CD8⁺ and CD4⁺ T cells for adoptive transfer for patients with cancer.

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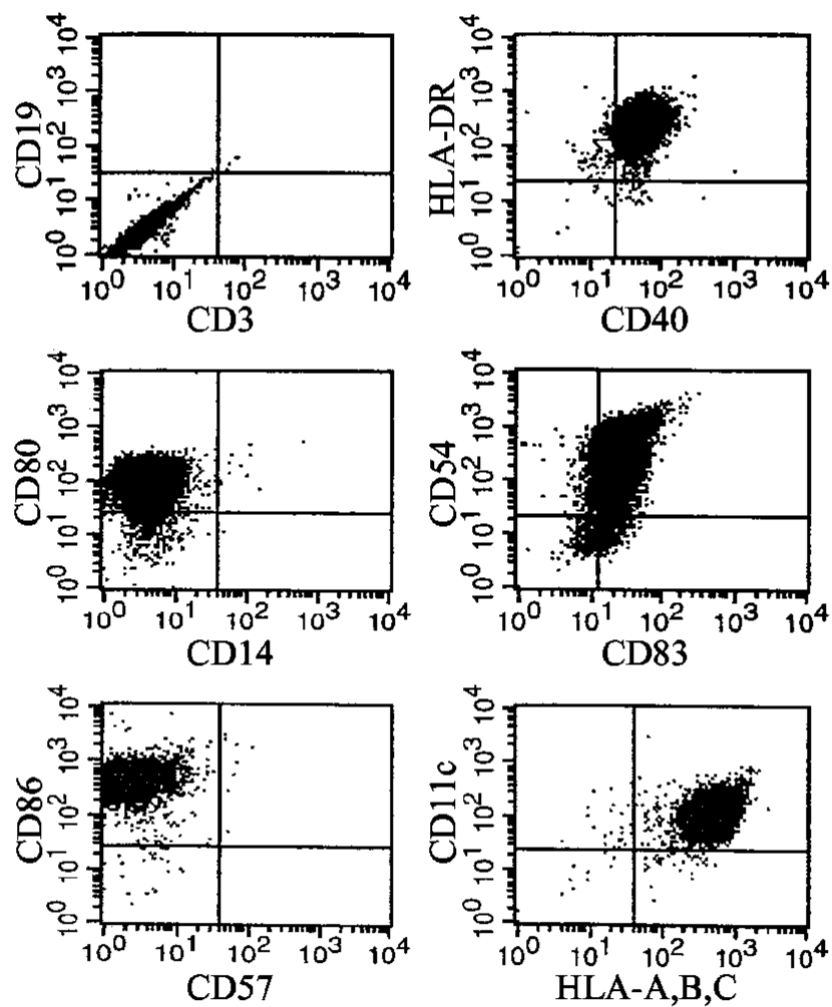


FIGURE 1. DC phenotype before electrofusion. DCs from donor 4 were stained with FITC- (*x*-axis; FL1-H) and PE- (*y*-axis; FL2-H) conjugated mAbs as indicated and analyzed by FACS.

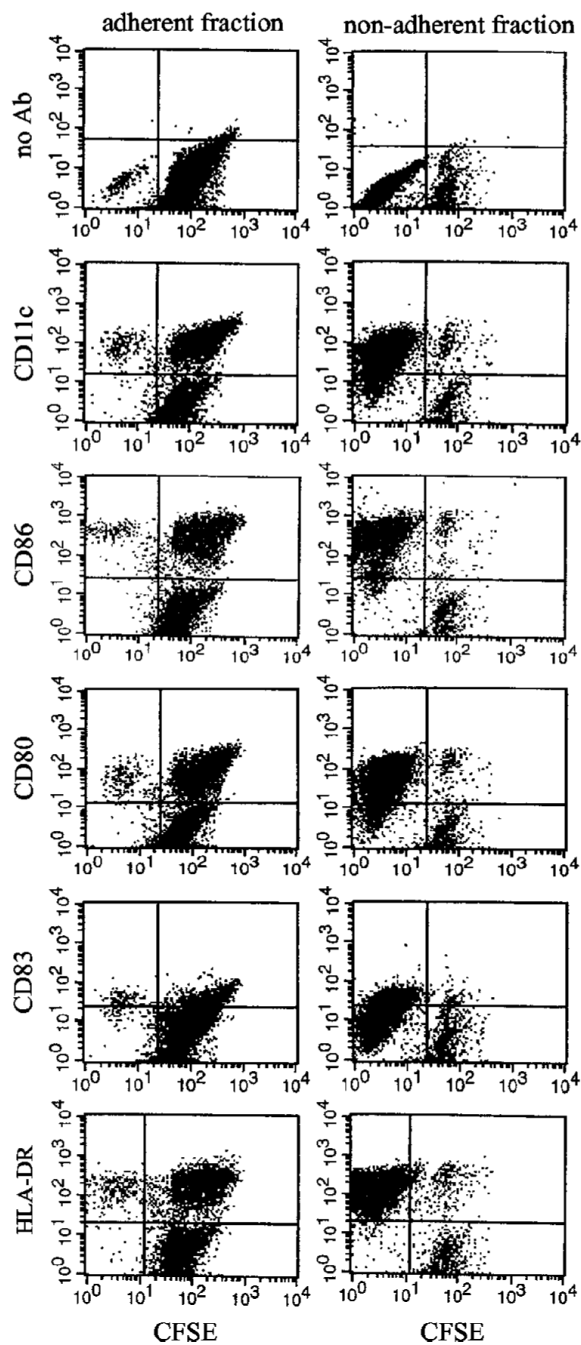


FIGURE 2.

FACS analyses of DC-TC hybrid cells generated by electrofusion. 888 melanoma cells were labeled with CFSE (x-axis; FL1-H) and were fused with DCs from donor 4. After overnight culture, both adherent and nonadherent cell populations were stained with PE- (y-axis; FL2-H) conjugated mAbs as indicated.

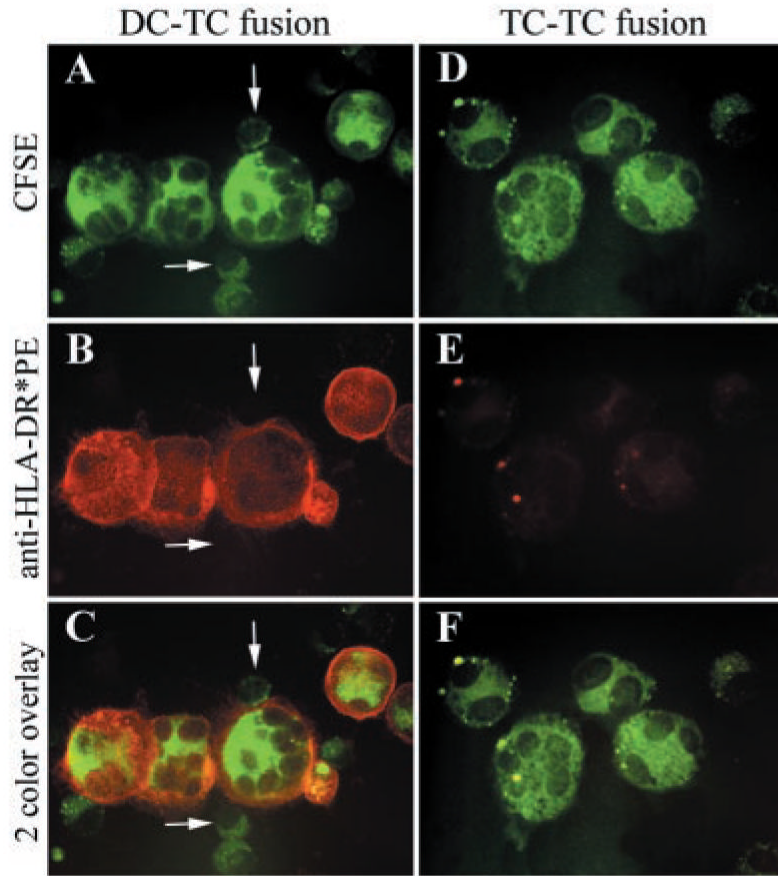
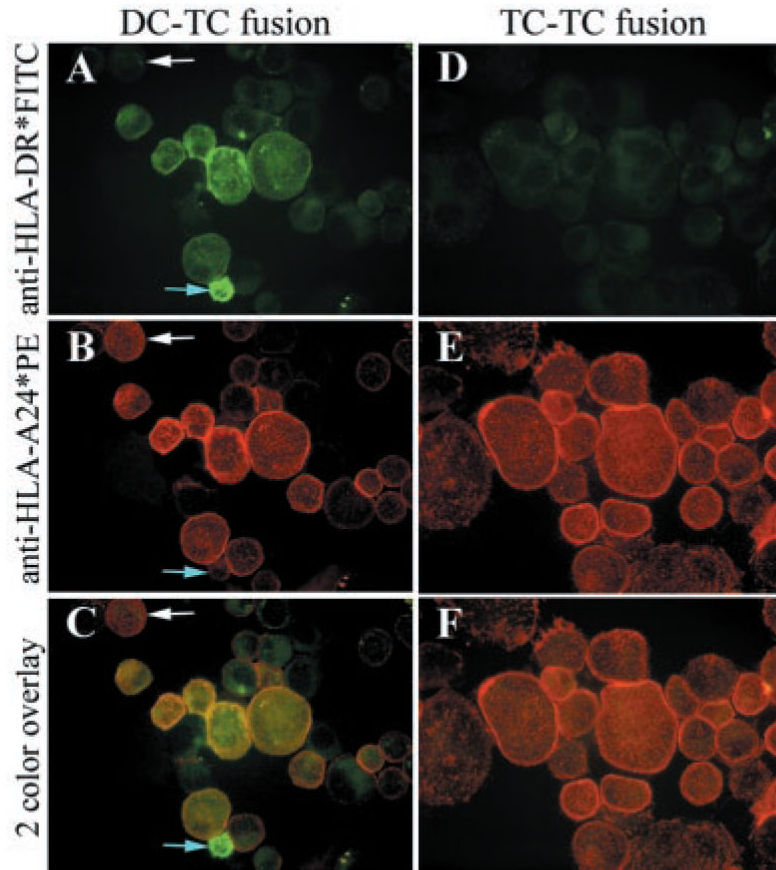


FIGURE 3.

Fluorescence micrographs of hybrid cells generated by electrofusion. *A–C*, 888 melanoma cells were labeled with CFSE (*A*) and were fused with DCs from donor 4. After overnight culture, the adherent cell population was stained with PE-conjugated anti-HLA-DR (*B*). Finally, a two-color overlay was prepared to enable visualization of double-positive cells (*C*). As estimated by FACS analysis, 29% of cells in the adherent fraction of DC-888mel fusion cells from donor 4 in this experiment were positive for both CFSE and HLA-DR. White arrows indicate melanoma cells that were only positive for CFSE. *D–F*, As a control, 888 melanoma cells were labeled with CFSE (*D*) and were fused in the absence of DCs. After overnight culture, the adherent cell population was stained with PE-conjugated anti-HLA-DR (*E*). Finally, a two-color overlay was prepared to enable visualization of double-positive cells (*F*).

**FIGURE 4.**

Fluorescence micrographs of hybrid cells generated by electrofusion. *A–C*, 888 melanoma cells (without prior CFSE labeling) were fused with DCs from donor 7. After overnight culture, the adherent cell population was concomitantly stained with FITC-conjugated anti-HLA-DR (*A*) and anti-HLA-A24 that was indirectly coupled to PE (*B*). Finally, a two-color overlay was prepared to enable visualization of double-positive cells (*C*). The white arrow indicates an individual tumor cell that only expressed HLA-A24, and the blue arrow indicates a single DC that only expressed HLA-DR. *D–F*, As a control, 888 melanoma cells (without prior CFSE staining) were fused in the absence of DCs. After overnight culture, the adherent cell population was concomitantly stained with FITC-conjugated anti-HLA-DR (*D*) and anti-HLA-A24 that was indirectly coupled to PE (*E*). Finally, a two-color overlay was prepared to enable visualization of double-positive cells (*F*).

Table I

Characteristics of Ag-reactive T cell lines and clones

T Cell Clone/Line	CD4/CD8	HLA Restriction Element	Peptide Specificity	Reference
TIL 1200	CD8	HLA-A*0201	gp100:154–162	38,39
CK3H6	CD8	HLA-A*0201	gp100:209–217	40
JR1E2	CD8	HLA-A*0201	gp100:280–288	40
MB4	CD8	HLA-A*0201	TRP2-6b:403–411	41
JB2F4	CD8	HLA-A*0201	MART-1:27–35	40
GA3D	CD8	HLA-A*0201	tyrosinase:368–377(370D)	42
TH1F2L	CD8	HLA-A*0201	NY-ESO-1:157–165	43
B104	CD4	HLA-DR β 1*0401	gp100:170–190	44
BR-B8	CD4	HLA-DR β 1*0701	gp100:44–59	45

Table II

Recognition of DCs loaded with gp100 protein^a

Target Cells	Preloaded Peptide ^b	Genetic Modification	Preloaded Protein ^c	CK3H6 (CD8)	TIL 1200 (CD8)	B104 (CD4)
Media	— ^d	—	—	151	61	39
F002Rmel CIITA	—	VSV-GFP	—	144	57	42
F002Rmel CIITA	—	VSV-gp100	—	10,989 ^e	8,258	>20,000
Donor 1 DC	gp100:209–217	—	—	102	44	38
	gp100:154–162	—	—	>20,000	nd	nd
	gp100:170–190	—	—	nd	328	nd
	—	Ad-MART-1	—	157	50	>20,000
	—	Ad-gp100	—	7,053	371	>20,000
	—	—	NY-ESO-1	801	65	624
	—	—	gp100	392	53	>20,000
	—	—	gp100 ⁻ lysate ^f	134	47	41
	—	—	gp100 ⁺ lysate ^g	112	49	>20,000
Donor 2 DC	—	—	—	116	43	43
	gp100:209–217	—	—	>20,000	nd	nd
	gp100:154–162	—	—	nd	350	nd
	gp100:170–190	—	—	166	52	>20,000
	—	Ad-MART-1	—	5,981	198	41
	—	Ad-gp100	—	142	51	8,088
	—	—	NY-ESO-1	134	48	160
	—	—	gp100	120	44	>20,000
	—	—	gp100 ⁻ lysate	122	52	40
	—	—	gp100 ⁺ lysate	113	46	>20,000
Donor 3 DC	—	—	—	>20,000	nd	38
	gp100:209–217	—	—	nd	nd	nd
	gp100:154–162	—	—	nd	292	nd
	gp100:170–190	—	—	255	70	>20,000
	—	Ad-MART-1	—	7,489	219	41
	—	Ad-gp100	—	426	81	6,413
	—	—	NY-ESO-1	148	53	423
	—	—	gp100	274	64	>20,000
	—	—	gp100 ⁻ lysate	142	56	50
	—	—	gp100 ⁺ lysate	—	—	>20,000

^aRecognition of DCs pulsed with recombinant gp100 protein or melanoma cell lysates or transduced with recombinant adenovirus encoding gp100 by HLA-A*0201 and HLA-DRβ1*0701-restricted, gp100-reactive T cell populations. IFN-γ secretion (picograms per milliliter) in 20 h coculture supernatants of target cells expressing both HLA-A*0201 and HLA-DRβ1*0701 with T lymphocytes. nd, not done.

^bDCs were preincubated with 50 μg/ml gp100:170–190, 1 μg/ml gp100:209–217, or 1 μg/ml gp100:154–162.

^cDCs were preincubated with recombinant proteins at 10 μg/ml or melanoma cell lysates at a ratio of 1 DC to 1 melanoma cell equivalent overnight at 37°C.

^d— indicates none.

^eUnderlined values indicate that IFN-γ secretion in response to DCs loaded with protein, peptide, or melanoma lysates, or adenovirally transduced DCs, or melanoma cell lines was ≥50 pg/ml and at least twice background with relevant control target cells.

^fThe gp100⁻ lysate was prepared from F002Rmel CIITA cells transduced with VSV-GFP.

⁸The gp100⁺ lysate was prepared from F002Rme1 CIITA cells transduced with VSV-gp100.

Table III
Recognition of DC-TC hybrids by HLA-A*0201-restricted, tumor-reactive T cell populations^a

Expt.	Target Cell	HLA-A2 Expression	Cell Description	TIL 1200	CK3H6	JR1E2	MB4	JB2F4	GA3D	TH1F2L
1	Media	-	None	254	8	0	33	0	178	14
	T2 + HBV:18-26(23Y)	+	Nonfused	180	2	0	14	20	93	312
	T2 + relevant peptide	+	Nonfused	4,355 ^b	>20,000	13,496	>20,000	5,572	6,049	>20,000
	888mel	-	Nonfused	156	0	6	34	8	33	21
	526mel CIITA	+	Nonfused	5,899	4,348	2,493	12,731	7,014	2,546	21
	624mel CIITA	+	Nonfused	5,708	8,918	9,581	>20,000	18,734	9,591	8,592
	Donor 2 DC-888mel ^c	DC ⁺ -mel ⁻	Fused adherent ^d	>2,000	3,323	1,553	266	1,577	5,773	68
	Donor 2 DC-888mel ^c	DC ⁺ -mel ⁻	Fused nonadherent	328	258	21	63	60	1767	136
	Donor 2 DC-888mel ^c	DC ⁺ -mel ⁻	Physically mixed	249	32	2	59	0	516	100
	Donor 4 DC-888mel ^c	DC ⁺ -mel ⁻	Fused adherent ^e	>2,000	4,148	1,252	190	775	6,050	60
	Donor 4 DC-888mel ^c	DC ⁺ -mel ⁻	Fused nonadherent	270	121	13	44	6	2,399	99
	888mel ^c	-	Fused adherent	238	14	18	37	0	22	31
	Media	-	None	245	13	8				
Donor 2 DC-888mel ^g	DC ⁺ -mel ⁻	Fused adherent	1,407	1,873	253					
Donor 5 DC-888mel ^h	DC ⁺ -mel ⁻	Fused adherent	276	16	19					

^aIFN- γ secretion (picograms per milliliter) in 20 h coculture supernatants of target cells with T lymphocytes. The data in each of the two panels were generated in separate experiments.

^bUnderlined values indicate that IFN- γ secretion in response to peptide-loaded T2 cells, HLA-A*0201⁺ melanoma cell lines, or hybrid cell populations was 50 pg/ml and at least twice background with any HLA-A*0201⁻ or Ag⁻ target cell (including a physical mixture of 888 melanoma cells and DCs).

^cFor these fused cell populations, 1×10^5 T cells were coincubated with 3.75×10^4 target cells.

^dIn the adherent fraction of DC-888mel fusion cells from donor 2 in this experiment, 48% of cells were positive for both CFSE and HLA-DR by FACS.

^eIn the adherent fraction of DC-888mel fusion cells from donor 4 in this experiment, 29% of cells were positive for both CFSE and HLA-DR by FACS.

^fIn the second experiment, 1×10^5 T cells were coincubated with 1.5×10^4 target cells.

^gIn the adherent fraction of DC-888mel fusion cells from donor 2 in this experiment, 19% of cells were positive for both CFSE and HLA-DR by FACS.

^hIn the adherent fraction of DC-888mel fusion cells from donor 5 in this experiment, 25% of cells were positive for both CFSE and HLA-DR by FACS.

Recognition of DC-TC hybrids by HLA-DRβ1*0401- and -DRβ1*0701-restricted, gp100-reactive T cell populations^a

Table IV

Expt.	Target Cell	HLA-DR4/DR7 Expression	Cell Description	BR-B8	B104
1	Media	DR4 ⁺ DR7 ⁻	None	11	0
	Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused adherent ^c	1,475 ^d	725
	Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused nonadherent	399	38
	Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Physical mixture	0	7
	Donor 4 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused adherent ^e	1,475	255
	Donor 4 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused nonadherent	243	29
	888mel ^b	DR4 ⁺ DR7 ⁻	Fused adherent	0	0
	Media	DR4 ⁺ DR7 ⁻	None	0	1
	Donor 2 DC-888mel ^g	DC ⁺⁺ -mel ⁻	Fused adherent	2,460	68
	Donor 5 DC-888mel ^h	DC ⁺⁺ -mel ⁻	Fused adherent	8	25
3	Media	DR4 ⁺ DR7 ⁻	None	0	10
	1088 EBV-B + Flu HA:306-324	DR4 ⁺ DR7 ⁻	Nonfused	4	
	1088 EBV-B + gp100:44-59	DR4 ⁺ DR7 ⁻	Nonfused	1,664	
	1978 EBV-B + IgK:188-201	DR4 ⁺ DR7 ⁺	Nonfused		19
	1978 EBV-B + gp100:170-190	DR4 ⁺ DR7 ⁺	Nonfused		7,541
	888mel	DR4 ⁺ DR7 ⁻	Nonfused	0	0
	F002Rmel CIITA-GFP	DR4 ⁺ DR7 ⁺	Nonfused	0	8
	F002Rmel CIITA-gp100	DR4 ⁺ DR7 ⁺	Nonfused	10	>20,000
	624mel CIITA	DR4 ⁺ DR7 ⁺	Nonfused	1,946	9,673
	1088mel CIITA	DR4 ⁺ DR7 ⁻	Nonfused	3,519	25
Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused adherent ^c	5,338	883	
Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Fused nonadherent	937	66	
Donor 2 DC-888mel ^b	DC ⁺⁺ -mel ⁻	Physical mixture	31	29	
888mel ^b	DR4 ⁺ DR7 ⁻	Fused adherent	0	0	

^aIFN-γ secretion (picograms per milliliter) in 20 h coculture supernatants of target cells with T lymphocytes. The data in each of the three panels were generated in separate experiments.

^bFor these fused cell populations, 1×10^5 T cells were coincubated with 3.75×10^4 target cells.

^cIn the adherent fraction of DC-888mel fusion cells from donor 2 in this experiment, 48% of cells were positive for both CFSE and HLA-DR by FACS.

^dUnderlined values indicate that IFN-γ secretion in response to peptide-loaded EBV-B cells, melanoma cell lines, or hybrid cell populations was 50 pg/ml and at least twice background with relevant control target cells (including a physical mixture of 888 melanoma cells and DCs).

^eIn the adherent fraction of DC-888mel fusion cells from donor 4 in this experiment, 29% of cells were positive for both CFSE and HLA-DR by FACS.

^fIn the second experiment, 1×10^5 T cells were coincubated with 1.5×10^4 target cells.

^gIn the adherent fraction of DC-888mel fusion cells from donor 2 in this experiment, 19% of cells were positive for both CFSE and HLA-DR by FACS.

^hIn the adherent fraction of DC-888mel fusion cells from donor 5 in this experiment, 25% of cells were positive for both CFSE and HLA-DR by FACS.