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Generation and Functional Assessment of Antigen-specific T Cells Stimulated by Fusions of Dendritic Cells and Allogeneic Breast Cancer Cells

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Abstract

We have reported that fusions of patient-derived dendritic cells (DC) and autologous breast cancer cells induce T-cell responses against autologous tumors. However, the preparation of fusion cells requires patient-derived tumor cells, and these are not always available in the clinical setting. In the present study, we explore an alternative approach to constructing DC-breast cancer fusion vaccine by using breast cancer-cell lines. DC generated from HLA-A*0201-positive donor were fused to HLA-A*0201⁺ allogeneic MCF7 breast cancer cells. These fusion cells co-expressed tumor-associated antigens and DC-derived costimulatory and MHC molecules. Both CD4 and CD8 T cells were activated by the fusion cells as demonstrated by the production of IFN- γ . The fusion cells induced strong antigen-specific CTL activity against their parent cells. The lysis of targets was restricted by HLA-A*0201, since killing was blocked by the anti-HLA-A2 mAb. Similar CTL activity against HLA-A*0201-positive targets was induced when T cells were cocultured with fusions of DC and HLA-A*0201-negative allogeneic BT20 breast cancer cells. In addition, administration of T cells stimulated by DC-breast cancer fusion cells regressed seven-day-old tumors and rendered mice free of disease up to 90 days. These results suggest that tumor-cell lines can be used as a fusion partner in the construction of DC-tumor fusion vaccine. Such fusion cells hold promise since they can be used as a vaccine for active immunotherapy or as stimulators to activate and expand T cells for adoptive immunotherapy.

Keywords

adoptive immunotherapy; allogeneic breast cancer cells; DC-breast cancer fusion cells; SCID mice

1. INTRODUCTION

Fusion of dendritic cells (DC) with tumor cells is a versatile approach in the creation of tumor vaccine [1]. Various fusion cells have been constructed by alternating the fusion partners, DC,

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and/or tumor cells. DC have been fused to autologous tumor cells [2–5]. In this case, the tumor antigens, known or unidentified, can be fully presented in the context of self HLA class I and class II molecules to autologous T cells. Allogeneic DC have been used to fuse with syngeneic or autologous tumor cells [3,6–8]. The advantage of this approach is that the fusion cells express both tumor-derived HLA class I molecules for the presentation of self MHC-restricted tumor peptide and allogeneic HLA class II molecules derived from the DC for direct stimulation of patient CD4 T cells [9]. We have not explored the third approach in DC-tumor fusions in which DC are fused with allogeneic tumor cells. The potential benefit of this approach is that the tumor antigen can be presented through the matched HLA molecules and alloreactive T cells can be induced against the unmatched HLA molecules. In addition, this approach can overcome one of the limiting factors in the application of fusion vaccine in the clinical setting by providing an unlimited source of tumor cells. In the present study, DC-derived from HLA-A*0201-positive human peripheral blood mononuclear cells (PBMC) were successfully fused to HLA-A*0201⁺ MCF7 breast cancer cells. The fusion cells expressed both tumor-derived HLA class I molecules, tumor-associated antigens MUC1 and Her-2/*neu*, and DC-derived costimulatory and HLA class I and class II molecules. Coculture of the fusion cells with T cells from the HLA-A*0201⁺ donors from whom DC were obtained resulted in the generation and expansion of antigen-specific cytotoxic T lymphocytes (CTL). The administration of CTL generated *ex vivo* by fusion cells eradicated large established tumors in SCID mice. In addition, HLA-A*0201-positive DC were fused to HLA-A2-negative BT20 breast cancer cells. The fusion cells were able to stimulate HLA-A*0201⁺ T cells to kill HLA-A*0201-positive and MUC1-positive tumor cells. The present study indicates that fusion of DC with either HLA-A*0201 matched or unmatched tumor cells is an effective approach for delivering tumor antigens to DC and inducing antigen-specific T cells.

2. MATERIALS AND METHODS

2.1. Cell lines

Human MCF7 breast carcinoma cells and OV-3 ovarian carcinoma cells (American Type Culture Collection (ATCC), Manassas, VA) were grown in DMEM medium; human BT20 breast carcinoma cells (ATCC) were grown in EMEM medium; human ZR-75 breast carcinoma cells and K562 cells (ATCC) were grown in RPMI medium 1640; human SK-BR3 breast carcinoma cells (ATCC) were grown in McCoy's medium. All media were supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin.

2.2. Preparation of DC and T cells

Peripheral blood mononuclear cells (PBMC) from HLA-A*0201⁺ healthy donors were isolated by Ficoll density-gradient centrifugation. The PBMC were suspended in RPMI medium 1640 supplemented with 10% human serum (Sigma Chemical Co., St. Louis, MO) for 30 min. The nonadherent cells were removed and cultured in 10U/ml IL-2 medium for further generation of the T-cell population (T cells were isolated by separation on a nylon-wool column). The adherent cells were cultured overnight. Then nonadherent and loosely adherent cells were collected and incubated for one week in RPMI medium 1640 with 1% human serum containing 1000 units/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme) and 500 units/ml IL-4 (Genzyme). The GM-CSF/IL-4-generated DC were used for fusion cells.

2.3. Cell fusion

DC were mixed with MCF7 or BT20 tumor cells at a 10:1 ratio and washed once with serum-free medium. The mixed cell pellets were gently resuspended in prewarmed 50% polyethylene glycol (PEG, Sigma) at room temperature for 5 min. The PEG solution was diluted by slow addition of serum-free RPMI 1640 medium. The cell pellets obtained after centrifugation at

1,000 rpm were resuspended in RPMI 1640 medium supplemented with 10% human serum and 500 U/ml GM-CSF, and incubated at 37°C for 7 days. At that time, DC-breast cancer fusion cells was loosely adherent to the culture dish, and the fusion cells were selected by gentle pipetting to collect the loosely adherent cells and, thereby, separated from tumor–tumor fusions or unfused tumor cells that were firmly attached to the dish. The fusion efficiency was determined by dual expression of tumor antigens MUC1 and/or Her-2/neu, and HLA class II molecules.

2.4. FACS analysis

Cells were washed with PBS and incubated for 40 min on ice with antibodies directed against MUC1 (HMPV, BD PharMingen), Her-2/*neu* (TA-1, Oncogene Research), MHC class I (W6/32), HLA-DR (TU36), CD83 (HB15e), CD80 (BB1/B7-1), CD86 (IT2.2) (BD PharMingen) and HLA-A2 (A2) or HLA-A11 (A11) (One Lambda, Canoga Park, CA). After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti-mouse IgG for 30 min on ice. Samples were then washed, fixed with 2% paraformaldehyde, and subjected to analysis by FACScan (Becton Dickinson).

2.5. Phenotype of T cells

PBMC from a healthy donor were isolated by Ficoll-gradient centrifugation. Nonadherent cells from PBMC at 5×10^7 /well in 24-well plates were cocultured with MCF7, DC mixed with MCF7, or DC-MCF7 fusion cells at 10:1 stimulator/responder ratio for 15 days. T cells were purified by nylon wool separation and stained with antibodies directed against CD3, CD4, CD8, $\alpha\beta$ -TCR and CD28 (BD PharMingen), and then with FITC-conjugated anti-mouse IgG staining. T cells were washed, fixed with 2% paraformaldehyde, and subjected to analysis by FACScan (Becton Dickinson).

2.6. Proliferation of T cells

DC, MCF7, or DC-MCF7 fusion cells were exposed to 30 Gy of ionizing radiation and added to T cells from HLA-A*0201⁺ donors from whom DC were obtained at various ratios (1:1–1:10) in 96-well U-bottom culture plates and incubated for 6 days. ³[H]thymidine uptake by T cells was measured 12 h after a pulse of 1 μ Ci per well (1 Ci = 37 GBq, New England Nuclear Corporation, Boston, MA). Tritium incorporation was quantified by liquid scintillation counting. All determinations were conducted in triplicate and expressed as the mean $\pm$ SE.

2.7. IFN- γ or IL-4 staining

To determine the presence of activated T cells, IFN- γ or IL-4 expression was assessed by intracellular staining with a human IFN- γ or IL-4 secretion assay kit according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). T cells were cocultured with DC-MCF7 fusion cells, DC mixed with MCF7 or T cells were treated with anti-CD3 mAb (1 μ g/ml) for 8 days and then collected for analysis of IFN- γ and IL-4 expression. In an independent study, T cells were cocultured with DC-BT20 fusion cells or DC mixed with BT20. Then T cells (1×10^7) were incubated with IFN- γ or IL-4 catch reagent for 5 min at 4°C, and 10 ml warm medium was added to the culture for 45 min at 37°C with shaking. After incubation, cells were labeled with PE-conjugated anti-human IFN- γ detection antibodies and further stained with FITC-anti-CD4 or anti-CD8 mAb (Miltenyi Biotec) for 20 min on ice. Cells were fixed and analyzed by flow cytometry.

2.8. Tetramer assay

Tetrameric assay of soluble class I MHC-peptide complexes were used to detect antigen-specific CTL [6]. Tetramer of HLA-A*0201-MUC1 peptide (STAPPVHNV) and irrelevant tetramer were purchased from PROIMMUNE (Oxford, UK). The tetramer staining was

performed according to the manufacturer's instructions. Briefly, the T cells from an HLA-A*0201⁺ donor from whom DC were obtained were cultured with DC-MCF7 or DC-BT20 fusion cells for up to 12 days. The T cells cocultured with DC mixed with MCF7, DC mixed with BT20 or anti-CD3 mAb (1 µg/ml) were used as controls. They were harvested and purified through nylon wool, then incubated with FITC-conjugated anti-human CD8 antibody for 40 min at 4°C. After washing, PE-conjugated HLA-A*0201/MUC1 tetramer or irrelevant tetramer was added to the cells for 60 min at room temperature. Cells were fixed after washing and analyzed by FACS using CellQuest software (BD Biosciences).

2.9. CTL assay

PBMC from HLA-A*0201⁺ donors from whom DC were obtained were cocultured with MCF7, DC mixed with MCF7, or DC-MCF7 fusion cells for 7–8 days in the presence of 10 units/ml human IL-2 (HuIL-2). The stimulated T cells were harvested by separation on a nylon wool column and used as effector cells in CTL assays. In an independent experiment, PBMC were cocultured with DC-BT20 fusion cells, DC mixed with BT20, or T cells treated with CD3 or medium alone for 7–8 days and then assayed for CTL activity. The targets included MCF7, SK-BR-3, ZR-75, OV-3 tumor cells, DC, or DC transfected with MUC1 mRNA (DC/MUC1) [10]. All targets were labeled with ⁵¹Cr for 60 min at 37°C. After washing to remove unincorporated isotope, the targets (2×10^4) were cocultured for 5 h at 37°C with various numbers of effector cells. In the antibody-blocking assays, the target cells were incubated with mAb W6/32 (anti-MHC class I) or A2 (anti-HLA-A2) for 30 min at 4°C before the addition of effector cells. The supernatants were collected and assayed for ⁵¹Cr release in a gamma counter. Spontaneous release of ⁵¹Cr was assessed by incubation of targets in the absence of effectors, and maximum or total release of ⁵¹Cr was determined by incubation of targets in 0.1% Triton X-100. Percentage of specific ⁵¹Cr release was calculated using the following equation:

$$\text{percent specific release} = [(\text{experimental} - \text{spontaneous}) / (\text{maximum} - \text{spontaneous})] \times 100$$

2.10. Adoptive immunotherapy

Four-week-old female CB-17/Icr SCID mice were purchased from Taconic Farms and maintained in a special pathogen-free facility. The SCID mice were inoculated s.c. with 7.5×10^6 MCF7 tumor cells in 150 µl matrigel (Becton Dickinson, Bedford, MD) and 50 µl sterile PBS. Seven days after tumor inoculation, the SCID mice were randomized into three groups and treated with T cells stimulated by (i) CD3 mAb (1 µg/ml); (ii) DC-MCF7; (iii) DC mixed with MCF7. One group of mice was inoculated with SK-BR3 tumor cells and treated with T cells stimulated by DC-MCF7. T cells (1.5×10^6) were injected into tumors three times at weekly intervals. The mice were followed up to 90 days and tumors were measured every other day. Tumor volume were calculated according to the following equation:

$$V = \text{length} \times (\text{width})^2 / 2.$$

3. RESULTS

3.1. Characterization of DC-tumor fusion cells

DC from HLA-A*0201⁺ donors were generated in GM-CSF/IL-4 medium for 5 days. The DC were fused with HLA-A*0201⁺ MCF7. To assess fusion, the fused cells were analyzed for the expression of tumor antigens and costimulatory molecules by flow cytometry. Figure 1 shows that DC generated from HLA-A*0201⁺ donor expressed HLA-A*0201, HLA-DR, CD86 (B7-1), CD80 (B7-2) and CD83 molecules and were negative for MUC1 and Her-2/*neu* tumor antigens, whereas MCF7 carcinoma cells expressed HLA-A*0201 and MUC1 and Her-2/*neu* tumor antigens and were negative for HLA-DR and costimulatory molecules. In contrast,

DC-MCF7 fusion cells expressed HLA-A*0201, MUC1, HLA-DR, CD86 (B7-1), CD80 (B7-2) and CD83 (Fig. 1A). To determine the fusion efficiency, DC-MCF7 fusion cells were stained with mAbs against MUC1 or Her-2/*neu* and HLA-DR or CD86, respectively. The fusion efficiency ranged from 34.4 to 60.9% after subtracting the double positive cells from DC mixed with tumor cells (Fig. 1B and C). These results indicate that the fusion cells possess the phenotypic properties of their parent cells.

3.2. Proliferation and characterization of T cells stimulated by HLA-A*0201-matched fusion cells

To determine the proliferation of T cells induced by fusion cells, DC-MCF7 fusion cells were cocultured for 6 days with T cells from the same donor from whom the DC were obtained. T cells cocultured with DC mixed with MCF7 or MCF7 alone were used as controls. T-cell proliferation was induced by fusion cells, and to a lesser extent, by DC mixed with MCF7 cancer cells (Fig. 2A). In contrast, no T-cell proliferation occurred when cells were cocultured with MCF7 breast cancer cells alone (Fig. 2A).

To characterize the proliferated T cells, their phenotype was analyzed by flow cytometry. The T cells expressed CD3, $\alpha\beta$ -TCR and CD28 after 15 days in culture (Fig. 2B). However, the numbers of CD4 or CD8 T cells activated by fusion cells or DC mixed with tumor cells were dissimilar. Coculture with fusion cells increased the population of CD8 T cells. Although CD8 T cells also increased after coculture with DC mixed with tumor cells, the magnitude was much less (Fig. 2B). These results suggest that the fusion process between DC and tumor cells enhances antigen-processing and -presentation through the MHC class I pathway to activate and/or maintain CD8 T cells.

3.3. Stimulation of IFN- γ secretion in T cells by DC-MCF7 fusion cells

Cytokine expression was analyzed to determine T-cell activation. T cells cocultured with DC-MCF7 were collected at indicated time points and analyzed for expression of IFN- γ and IL-4. IFN- γ -expressing CD4 and CD8 T cells appeared on Day 6 and persisted to Day 9 (Fig. 2C). IL-4 secretion was detected in a few T cells stimulated by DC-MCF7 fusion cells on Day 9 (Fig. 2C). In contrast, few T cells stimulated by DC mixed with MCF7 cells or anti-CD3 mAb produced IFN- γ (Fig. 2D and E). Moreover, MUC1-peptide-specific CTL were detected in T cells stimulated by DC-MCF7 fusion cells (Fig. 2C). In contrast, no positive T cells were detected when an irrelevant tetramer was used (data not shown). These results indicate that fusion cells have the ability to stimulate both CD4 and CD8 T cells and generate tumor-antigen-specific T cells.

3.4. Generation of antigen-specific cytotoxic T cells by HLA-A*0201 matched DC-MCF7 fusion cells

Chromium-release assay was used to assess the induction of CTL response. CTL stimulated by DC-MCF7 fusion cells killed 90% of MCF7 cells. In contrast, only 20% and 18% lysis was observed against SK-BR3 or K562 targets, respectively (Fig. 3A). MCF7 carcinoma cells were not killed by T cells induced by CD3 mAb or treated with medium. DC mixed with MCF7 cells induced 30% CTL activities against MCF7 tumor cells (Fig. 3A). To determine the antigen-specific and HLA-restricted tumor lysis by CTL, multiple tumor targets positive for either HLA-A*0201 or HLA-A*1101 were used (Fig. 3B). CTL stimulated by DC-MCF7 showed 88.7%, 50.12% and 40.5% lysis against MCF7 (HLA-A*0201), OV-3 (HLA-A*0201) and autologous DC transfected with MUC1-RNA targets (DC/MUC1), respectively (Fig. 3C). In contrast, there was much lower CTL activity against HLA-A*0201⁻ SK-BR3, ZR-75, BT20, or K562 targets and background CTL activity against autologous DC. Moreover, the lysis of MCF7, OV-3 and DC/MUC1 was significantly reduced after the targets were pre-incubated with anti-MHC class I mAb or anti-HLA-A2 mAb (Fig. 3C). Taken together, these results

indicate that the CTL stimulated by DC-MCF7 fusion cells are antigen-specific and HLA-A*0201 restricted.

3.5. Adoptive immunotherapy of established tumor with ex vivo generated CTL by HLA-A*0201 matched fusion cells

To determine the efficacy of CTL stimulated by DC-MCF7 fusion cells, we established a tumor model in SCID mice by inoculation with MCF7 or SK-BR3 breast cancer cells. Seven days after inoculation, the mice were treated with HLA-A*0201⁺ CTL induced by DC-MCF7 fusion cells. DC-MCF7 stimulated T cells regressed the tumors completely and the mice remained free of MCF7 tumor growth in the entire follow-up period (Fig. 4A). In contrast, progressive MCF7 tumor growth was observed in mice treated with T cells stimulated by anti-CD3 mAb or by DC mixed with MCF7 (Fig. 4A). Although CTL stimulated by DC-MCF7 fusion cells show 20% lysis of SK-BR3 tumors, they were not enough to control the progression of tumors (Fig. 4A and B). Figure 4B shows the mice after T-cell treatment. Only mice bearing MCF7 tumors were free of tumor after treatment with T cells stimulated by DC-MCF7 fusion cells. The results indicate the therapeutic potency of the T cells stimulated by HLA-matched DC-tumor fusion cells; these T cells can eradicate 7-day-old tumors.

3.6. Activation of HLA-A*0201-positive T cells by fusion of DC from HLA-A*0201 donor with HLA-A*0201-negative breast cancer cells

The previous data indicate that HLA-A*0201 matched DC-tumor fusion cells are able to present HLA-A*0201-restricted MUC1 epitopes to induce antigen-specific T cells. We do not know, however, whether only HLA-matched DC and tumor cells are able to induce antigen-specific T cells. To address this issue, we fused HLA-A*0201⁺ DC with HLA-A*0201⁻ BT-20 breast cancer cells (Fig. 5). Coculture of HLA-A*0201⁺ T cells from the same donor from whom the DC were generated with DC-BT20 fusion cells resulted in the expression of IFN- γ , but not IL-4, in some CD4 and CD8 T cells on Day 6 and 9 (Fig. 5A). Few T cells cocultured with DC mixed with BT20 tumor cells expressed IFN- γ (Fig. 5B). In addition, MUC1-peptide-specific CTL were detected in T cells stimulated by DC-BT20 fusions, but not by DC mixed with BT-20 cells (Fig. 5A and B). The CTL induced by DC-BT20 fusion cells were functional in the lysis of HLA-A*0201⁺ MCF7, or OV-3, but not of K562 targets (Fig. 5C). The CTL were significantly decreased by pretreatment of targets with W6/32 (Fig. 5D), indicating HLA class I restriction. These results indicate that fusions of HLA-A*0201⁺ DC and HLA-A*0201⁻ BT20 tumor cells were able to present tumor antigen to HLA-A*0201⁺ T cells regardless of HLA match.

4. DISCUSSION

Ex vivo induction and expansion of tumor-specific T cells for adoptive use are the subject of intensive investigation. Tumor-infiltrating lymphocytes (TIL) were the focus of studies [11–13]. Although the efficacy of TIL is questioned in the early study [14], adoptive transfer of TIL with co-administration of IL-2 to patients with melanoma met with some success [15]. However, the use of high dose of IL-2 in adoptive immunotherapy with TIL caused considerable toxicity to the host [16]. Recent use of non-myeloablative, but lymphodepleting systemic chemotherapy as a preconditioning before adoptive transfer of TIL has significantly improved the efficacy of the adoptive immunotherapy [17,18]. This finding, however, is limited to melanoma since tumor-infiltrated T cells are readily isolated from melanoma samples and can then be expanded in ex vivo to therapeutic levels and transferred back into the patient. Thus, alternative sources of tumor-reactive T cells with enhanced capability of tumor-cell destruction have been pursued. Tumor-specific CTL have been generated from peripheral blood lymphocytes and lymph node cells with a variety of stimulators [2,19–22]. In our previous studies, tumor cells derived from patients with ovarian or breast carcinoma and fused

with autologous or allogeneic DC induced antigen-specific CTL against autologous cancer cells [2,3]. The present study extends the previous findings by demonstrating: (i) fusion of DC with HLA-A*0201-matched or unmatched allogeneic breast cancer cell lines, (ii) induction of antigen-specific CTL by HLA-A*0201-matched or unmatched fusion cells, (iii) therapeutic efficacy of CTL in eradicating 7-day-old established tumors in SCID mice. These experiments provide further support that fusion of DC with tumor cells is a versatile approach in antitumor immunity. DC-tumor fusion cells can be used as a vaccine to activate presumably existing, yet limited, tumor-specific CTL precursors in the tumor-bearing host. Alternatively, the fusion cells can be used as stimulators to induce and expand ex vivo tumor-specific CTL to therapeutic numbers; then these CTL can be transferred to the tumor-bearing host for adoptive immunotherapy. Such fusion-cell-based active and adoptive immunotherapy, used in combination or independently, may represent a promising strategy in the treatment of cancer.

The advantage of using autologous tumor cells is that the whole set of tumor antigens can be presented in the context of self-HLA. However, autologous tumor cells may not be obtainable if surgery is not a component of the treatment. Tumor biopsy, even if obtained, may not provide sufficient numbers of viable tumor cells due to the lengthy culture time and potential contamination. An alternative approach may be the use of established tumor-cell lines. The basis for using tumor-cell lines is that some antigens such as MUC1 are shared by most of tumors. The present study provides evidence to support such a strategy.

How the fusion cells assemble and present the HLA-A*0201-restricted peptide complexes is unclear. One possibility is that the peptide is complexed with HLA-A*0201 molecules of tumor origin and the complexes are simply transferred to and presented by the fusion cells.

Alternatively, the tumor cells may provide tumor antigen and the fusion process simply facilitates the delivery of tumor antigen to the efficient antigen-processing and -presentation machinery of DC. Then the tumor antigen is presented in the context of MHC molecules derived from DC. Initially, we fused DC from HLA-A*0201⁺ donor to MCF7 cancer cells in the hope that tumor antigen can be presented through the matched HLA alleles. Indeed, coculture of PBMC from the same donor from whom DC were obtained with DC-MCF7 fusion cells induced strong CTL activity against MCF7 cancer cells. The cytotoxicity against MCF7 cells was abolished when the targets were treated with W6/32 or anti-HLA-A2 mAb, suggesting the killing was HLA class I- or HLA-A*0201-restricted. To confirm the antigen-specificity of the CTL, DC from the same donor transfected with MUC1 RNA were used as an autologous target. We observed moderate killing to MUC1-expressing DC, which was blocked by anti-HLA class I or anti-HLA-A2 mAb. In contrast, there was little lysis of autologous DC. We observed higher CTL activity against MCF7 tumor cells than against DC transfected with MUC1 antigen. One possible explanation is that DC-MCF7 fusion cells induced CTL against not only MUC1 but also other unidentified antigens expressed by MCF7 tumor cells. Alternatively, both MUC1-specific and alloreactive CTL were induced by DC-MCF7, and the combination of antigen-specific and alloreactive CTL accounted for the higher level of CTL activity.

MUC1-specific CTL in the murine model is MHC class I-restricted [23–25]. However, in humans, initial reports indicate that coculture of draining lymph node cells from cancer patients with MUC1⁺ human cancer cell lines expressing different HLA alleles resulted in the induction of MUC1-specific and MHC-unrestricted CTL [26,27]. Domenech et al. [28] identified an HLA-A*1101-restricted epitope from the tandem repeat of MUC1. More recently, HLA-A*0201-restricted MUC1-specific CTL were induced or identified in HLA-A*0201 transgenic mice [29] and in humans [30]. In the present study, coculture of PBMC with DC-MCF7 fusion cells stimulated MUC1-specific and HLA-A*0201-restricted CTL. However, there was 20% lysis against SK-BR-3 which express HLA-A*1101 and low level of MUC1. Interestingly, SK-BR-3 lysis is blocked by the anti-MHC I mAb, but not by HLA-A2 mAb (Fig. 3C). One possibility is that shared MHC molecules other than A*0201 may exist between the donor cells

and SK-BR-3 tumor cells. Taken together, our results show that HLA-A*0201 is the dominant restriction element in T cells stimulated by DC-tumor fusion cells. The dominant HLA-A*0201 restriction of CTL was further confirmed by the in vivo study. Injection of CTL eliminated 7-day-old HLA-A*0201⁺ and MUC1⁺ tumor cells. The 20% lysis against SK-BR-3 is not enough to regress the tumors.

After fusing HLA-A*0201⁺ DC with HLA-A*0201⁻ tumor cells, we observed that HLA-A*0201⁺ T cells stimulated by DC-BT20 fusion cells were able to kill breast or ovarian tumor targets with shared tumor antigen, MUC1, at a level similar to that induced by HLA-A*0201 matched DC-MCF7. These data indicate that the tumor antigens delivered to DC by fusion were processed and presented in the context of MHC molecules of DC origin. Therefore, DC and tumor cells need not be matched. The CTL stimulated by DC-MCF7 or DC-BT20 fusion cells exhibited a relatively high level of killing of HLA-A*0201 and MUC1-positive ovarian cancer cell line, OV3. This finding may have significant implication in that a universal vaccine based on common or shared tumor antigen may be feasible. Fusions of DC with HLA-A*0201-matched or unmatched allogeneic tumor cells can efficiently stimulate antigen-specific and HLA-A*0201-restricted CTL.

DC-tumor fusion cells have the ability to activate both CD4 and CD8 T cells [31] which are involved in antitumor immunity. In the present study, CD4 and CD8 T cells stimulated by fusion cells produced IFN- γ . The T cells cultured for 10 days were used for adoptive immunotherapy. These T cells were potent effector cells since 7-day-old tumors were cured by activated T cells alone without cytokine or adjuvant. The data demonstrate the feasibility of using tumor-cell lines for the construction of DC-tumor fusion vaccine and hold promise for fusion-cell-stimulated adoptive T-cell immunotherapy.

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Figure 1. Characterization of DC-tumor fusion cells

(A) DC from HLA-A*0201⁺ donor, MCF7 breast carcinoma cells, and DC-MCF7 fusion cells were stained with indicated mAb. (B and C) DC, MCF7, DC-MCF7 fusion cells and DC mixed with MCF7 tumor cells were cultured for 8 days, then double-stained with FITC-anti-MUC1 (HMPV) and PE-anti-HLA-DR (TU36) or PE-anti-CD86 (IT2.2) mAb (B), or FITC-anti-Her-2/neu (TA-1) and PE-anti-HLA-DR (TU36) or PE-anti-CD86 (IT2.2) mAb (C). Phenotype of cells was analyzed by FACS analysis.

Figure 2. Phenotypic and functional analysis of T cells stimulated by HLA-A*0201 matched DC-MCF7

T cells from HLA-A*0201⁺ donor were isolated by Ficoll density centrifugation and enriched by collection on nylon wool column. (A) Proliferation of T cells cocultured with DC-MCF7 (●), irradiated MCF-7 (○) or DC mixed with MCF7 (□). T cells were cocultured with above cells at indicated cell ratios in 10% human serum RPMI medium for 6 days. Cells were pulsed with 1 μCi ^3H -thymidine per well for 12 h, and ^3H -thymidine uptake was measured to determine T-cell proliferation. Results are expressed as mean $\pm$ SD of three replicates. (B) T cells from HLA-A*0201⁺ donor were cocultured with DC-MCF7, DC mixed with MCF7 cells, stimulated by anti-CD3 mAb or medium alone for 15 days, and stained with indicated antibodies. Phenotype of T cells was analyzed by flow cytometry. (C–E) Detection of IFN- γ and IL-4 secretion in T cells stimulated by DC-MCF7 fusion cells. T cells from HLA-A*0201⁺ donor were stimulated for 9–12 days with DC-MCF7 (C), DC mixed with MCF7 cells (D) or anti-CD3 mAb (1 $\mu\text{g}/\text{ml}$) (E). T cells were collected at indicated time, purified by passage through nylon wool, and stained for expression of IFN- γ and IL-4 in CD4 and CD8 T

cells. HLA-A*0201-MUC1 tetramer was used to measure antigen-specific CTL from HLA-A*0201⁺ donor stimulated by DC-MCF7, DC mixed with MCF7, or anti-CD3 mAb. Results represent three experiments.

Figure 3. Stimulation of antigen-specific CTL by fusion cells

(A) T cells from HLA-A*0201⁺ donor were cocultured with DC-MCF7 for one week. T cells cultured with anti-CD3 mAb, DC mixed with MCF7, or medium alone were used as controls. T cells were purified by passage through nylon wool and were cocultured with ⁵¹Cr-labeled MCF7 (○ HLA-A*0201⁺), SK-BR3 (△, HLA-A*1101⁺), or K562 (□, HLA-A*0201⁻ or A*1101⁻) cells at indicated E:T ratios. Percentage of cytotoxicity was determined by ⁵¹Cr release assay. (B) Targets were stained with indicated mAb and analyzed by flow cytometry. (C) T cells from HLA-A*0201⁺ donor were stimulated with DC-MCF7 for one week and CTL activity against indicated targets (black bar) was measured at 60:1 E:T ratio. In antibody-blocking assay, targets were pre-incubated for 1 h at 37°C with anti-MHC class I mAb (W6/32,

1:100 dilution, hatched bar) or anti-HLA-A2 mAb (A2, 1:100 dilution, dot bar), labeled with ^{51}Cr , and then cocultured with T cells. Percentage of cytotoxicity was determined by ^{51}Cr release assay. Results are expressed as the mean $\pm$ SD of three replicates.

Figure 4. Adoptive therapy of tumor-bearing mice with T cells stimulated ex vivo by DC-MCF7 (A) Adult SCID mice (n = 6/group) were inoculated s.c. with 7.5×10^6 MCF7 (●, △ or □) or SK-BR3 (○) breast carcinoma cells. Seven days after tumor inoculation, mice were treated with 1.5×10^7 T cells from HLA-A*0201⁺ donor stimulated by DC-MCF7 (● or ○) three times at weekly intervals. Mice receiving HLA-A*0201⁺ T cells stimulated by anti-CD3 mAb (△) or DC mixed with MCF7 (□) were used as controls. Mice were followed up to 90 days and tumors were measured every other day. (B) Photograph of SCID mice after adoptive therapy with T cells stimulated by DC-MCF7 (left panel), DC mixed with MCF7 (right panel), or anti-CD3 mAb (second from right). Mice bearing SK-BR3 tumor were treated with T cells stimulated by DC-MCF7 (second from left).

Figure 5. Induction of CTL by DC from HLA-A*0201⁺ donor fused with HLA-A*0201⁻ BT20 breast cancer cells

T cells from HLA-A*0201⁺ donor were cocultured with DC-BT20 (A) or DC mixed with BT20 tumor cells (B) and collected on indicated days. T cells were purified by passage through nylon wool and analyzed for intracellular IFN- γ secretion in CD4 and CD8 T cells. CD8 T cells were analyzed for HLA-A*0201-MUC1 tetramer. (C) T cells from HLA-A*0201⁺ donor were cocultured with DC-BT20 for one week. T cells were purified and cocultured with ⁵¹Cr-labeled MCF7 (□, HLA-A*0201⁺), OV-3 (Δ, HLA-A*0201⁺) or K562 (○, HLA-A*0201⁻) cells at indicated E:T ratios. Percentage of cytotoxicity was determined by ⁵¹Cr release. (D) T cells from HLA-A*0201⁺ donor were stimulated with DC-BT20 for one week and CTL activity

against indicated targets (black bar) was analyzed at 60:1 E:T ratio. In antibody-blocking assay, targets were pre-incubated for 1 h at 37°C with anti-MHC class I mAb (W6/32, 1:100 dilution, hatched bar), labeled with ^{51}Cr , and then cocultured with T cells. Percentage of cytotoxicity was determined by ^{51}Cr release assay. Results are expressed as mean $\pm$ SD of three replicates.