

## Dendritic Cells Fused with Allogeneic Colorectal Cancer Cell Line Present Multiple Colorectal Cancer – Specific Antigens and Induce Antitumor Immunity against Autologous Tumor Cells

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**Abstract** The aim of antitumor immunotherapy is to induce CTL responses against autologous tumors. Previous work has shown that fusion of human dendritic cells and autologous tumor cells induce CTL responses against autologous tumor cells *in vitro*. However, in the clinical setting of patients with colorectal carcinoma, a major difficulty is the preparation of sufficient amounts of autologous tumor cells. In the present study, autologous dendritic cells from patients with colorectal carcinoma were fused to allogeneic colorectal tumor cell line, COLM-6 (HLA-A2<sup>-</sup>/HLA-24<sup>-</sup>), carcinoembryonic antigen (CEA)<sup>+</sup>, and MUC1<sup>+</sup> as an alternative strategy to deliver shared colorectal carcinoma antigens to dendritic cells. Stimulation of autologous T cells by the fusion cells generated with autologous dendritic cells (HLA-A2<sup>+</sup> and/or HLA-A24<sup>+</sup>) and allogeneic COLM-6 resulted in MHC class I – and MHC class II – restricted proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, high levels of IFN- $\gamma$  production in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and the simultaneous induction of CEA- and MUC1-specific CTL responses restricted by HLA-A2 and/or HLA-A24. Finally, CTL induced by dendritic cell/allogeneic COLM-6 fusion cells were able to kill autologous colorectal carcinoma by HLA-A2- and/or HLA-A24-restricted mechanisms. The demonstration of CTL activity against shared tumor-associated antigens using an allogeneic tumor cell line, COLM-6, provides that the presence of alloantigens does not prevent the development of CTL with activity against autologous colorectal carcinoma cells. The fusion of allogeneic colorectal carcinoma cell line and autologous dendritic cells could have potential applicability to the field of antitumor immunotherapy through the cross-priming against shared tumor antigens and provides a platform for adoptive immunotherapy.

Dendritic cells are potent professional antigen-presenting cells able to induce primary immune responses (1, 2). The evidence for their ability to act as natural adjuvants in the induction of antitumor-specific CTL in murine and human models is now overwhelming. Dendritic cells capture and process antigens into peptides and present them through MHC classes I and II. Thus, dendritic cells are capable of stimulating both CD4<sup>+</sup> Th

cells and CD8<sup>+</sup> T cells (3, 4). Different strategies have been developed to load dendritic cells with tumor-associated antigens (TAA), including synthetic peptides derived from the known antigens, tumor RNA, tumor lysates, and dying tumor cells to induce antigen-specific immune responses (5–12). However, production of patient-specific cancer vaccines has currently been addressed in clinical trials; a major drawback of this strategy comes from a limited number of known tumor peptides available in many HLA contexts. Furthermore, dendritic cells pulsed with antigen-specific peptides were used in clinical trials for patients with colorectal carcinoma and other cancer patients, but results showed that clinical responses was found in a small number of patients (13–15).

An alternative strategy for inducing antitumor immunity is the use of fusion cells between dendritic cells and tumor cells (16). In this approach, multiple TAAs, including those yet unidentified, are endogenously processed and presented by MHC class I and II pathways in the context of costimulatory signals (17–19). In clinical study, patients with melanoma, glioma, or renal cell carcinoma immunized with autologous dendritic cell/tumor fusion cells were associated with immunologic and clinical responses (20–22). However, in the clinical setting of the patients with colorectal carcinoma, a major difficulty for the fusion vaccine is the preparation of sufficient amounts of autologous tumor cells. The specimen of

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Received 6/20/05; revised 8/6/05; accepted 8/10/05.

**Grant support:** Ministry of Education, Cultures, Sports, Science and Technology of Japan grants-in-aid for scientific research and Bio-Venture Research Fund Project Aid and Japanese Society of Gastroenterology.

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doi:10.1158/1078-0432.CCR-05-1330

colorectal carcinoma from primary lesion may not provide sufficient numbers of viable tumor cells due to the length of culture time and potential contamination of bacteria and fungus. In recent studies, hybrid cells generated by fusing dendritic cells from healthy donor with allogeneic tumor cell line have induced CTL responses against the allogeneic tumor cells used for fusion (23, 24). However, little is known about the nature of CTL induced by dendritic cell/allogeneic tumor fusion cells against autologous tumor cells.

Here, we have explored whether fusion cells generated by fusing autologous dendritic cells from patients with colorectal carcinoma and allogeneic colorectal carcinoma cell line, COLM-6, as a source of TAAs would permit T-cell priming and selection of CTL against autologous colorectal carcinoma cells. We show that autologous dendritic cells fused with allogeneic COLM-6 cells stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells and induce CTL responses that lyse autologous colorectal carcinoma cells and allogeneic tumor cell lines positive for the carcinoembryonic antigen (CEA) and/or MUC1 in HLA-A2 and/or HLA-A24 restriction manner. This demonstration of cross-priming against shared TAAs opens the possibility of using allogeneic tumor cell lines to deliver TAAs to dendritic cells for dendritic cell/tumor fusion vaccination protocols.

## Materials and Methods

**Cell lines.** Colorectal carcinoma cell lines obtained from primary lesion (COLP-2 and COLP-12), hepatic metastasis lesion (COLM-6 and COLM-7), lung metastasis lesion (COLM-21), and ovarian cancer cell line (OVAP-1) were established in the Saitama Cancer Center.<sup>7</sup> K562, breast cancer cell line MCF-7, lymphoma cell line Jurkat, and T2 cells were from the American Type Culture Collection (Rockville, MD). COLP-2, COLP-12, COLM-6, COLM-7, COLM-21, and OVAP-1 were maintained in TIL Media I medium (IBL, Gunma-ken, Japan); K562 and MCF-7 were maintained in DMEM; Jurkat cells were maintained in RPMI 1640; T2 cells were maintained in Iscove's modified Dulbecco's medium. All media were supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin.

**Generation of immature autologous monocyte-derived dendritic cells.** Monocyte-derived immature dendritic cells from a healthy volunteer or patients with colorectal carcinoma (institutional review board) were generated by Ficoll density-gradient centrifugation. Briefly, peripheral blood mononuclear cells (PBMC) were incubated in tissue culture flask at 37°C for 30 minutes in RPMI 1640 containing 1% heat-inactivated autologous serum. After incubation, nonadherent cells were removed, and adherent PBMCs were cultured in the presence of 1% heat-inactivated autologous serum/RPMI overnight. The loosely adherent cells were collected on the next day and placed in RPMI 1640 containing 1% heat-inactivated autologous serum, 1,000 units/mL granulocyte macrophage colony-stimulating factor (Pepro Tech, Rocky Hill, NJ), and 500 units/mL interleukin-4 (IL-4; Diaclone Research, Boulevard Fleming, France) for 5 to 7 days. Immature autologous monocyte-derived dendritic cells were harvested from the nonadherent and loosely adherent cells. The firmly adherent monocytes were harvested after treatment with trypsin and used as an autologous target for the CTL assay.

**Preparation of tumor cells.** Specimens from resected colorectal carcinoma metastatic lesions in the liver ( $n = 4$ ), lungs ( $n = 1$ ), lymph nodes ( $n = 1$ ), or primary lesion ( $n = 2$ ) were obtained with the approval of our institutional review board (Table 1). Solid tumors were

processed to cell suspension by collagenase (1 mg/mL), and colorectal carcinoma cells were isolated. Colorectal carcinoma cells were maintained in TIL Media I medium supplemented with 10% heat-inactivated autologous serum, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin in an appropriate flask based on volume until use as fusion cell preparations and targets for CTL assay.

**Fusion of immature dendritic cells and tumor cells.** Purified autologous immature dendritic cells were mixed with autologous colorectal carcinoma cells or allogeneic colorectal carcinoma cell line (COLM-6) at a ratio of 10:1 in serum-free prewarmed RPMI 1640. Mixed cell pellet were gently resuspended in prewarmed 50% polyethylene glycol (molecular weight = 1,450)/DMSO solution (Sigma-Aldrich, St. Louis, MO; 1 mL per  $5 \times 10^6$  cells) for 3 to 5 minutes at room temperature. Subsequently, the polyethylene glycol solution was diluted by slow addition and mixing of 1, 2, 4, 8, and 16 mL of serum-free prewarmed medium until 50 mL. Cell pellets obtained after centrifuge at 1,000 rpm were resuspended in RPMI 1640 supplemented with 10% autologous heat-inactivated serum and 500 units/mL granulocyte macrophage colony-stimulating factor and cultured in a 5% CO<sub>2</sub> atmosphere at 37°C for 5 to 7 days. By this time, each dendritic cell/tumor fusion cell was integrated into a single entity and was loosely adherent to the culture dish. Unfused tumor cells grow firmly attached to the plates, whereas dendritic cell/tumor fusion cells grow loosely in the wells and are suspended in medium. Dendritic cell/tumor fusion cells were selected and purified by gentle pipetting, and firmly attached tumor cells were discarded (25).

**Phenotype analysis.** Cells were washed and incubated with FITC-conjugated antibody against MUC1 (HMPV, BD PharMingen, San Diego, CA), CEA (B1.1, BD PharMingen), MHC class I (W6/32), MHC class II (HLA-DR), B7-1 (CD80), B7-2 (CD86; BD PharMingen), and HLA-A2 or HLA-A24 (One Lambda, Canoga Park, CA) for 1 hour on ice. After washing with cold PBS, cells were fixed with 2% paraformaldehyde. For analysis of dual expression, cells were incubated with FITC-conjugated anti-CEA or MUC1, washed with PBS, and then incubated with phycoerythrin-conjugated anti-HLA-DR, CD86, HLA-A2, or HLA-A24 for 1 hour at 4°C. Cells were washed, fixed, and analyzed by FACScan (Becton Dickinson, Mountain View, CA) with CellQuest analysis software.

**T-cell proliferation assay.** Nonadherent PBMCs were cocultured with dendritic cell/tumor fusion cells or dendritic cells mixed with tumor cells at a ratio of 10:1 in the presence of 20 units/mL human IL-2 (Shionogi, Osaka, Japan) and were purified through nylon wool to remove antigen-presenting cells and B cells. T-cell proliferation was done by Cell Titer 96 nonradioactive cell proliferation assay kit according to the protocol (Promega, Madison, WI). CD4<sup>+</sup> or CD8<sup>+</sup> T cells were isolated from purified T cells using MACS CD4 or CD8 Microbeads, respectively, according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA) on day 0. The purity of selected T-cell populations was in all cases >97% by flow cytometry analysis using FACScan. Whole T cells, purified CD4<sup>+</sup> T cells, or purified CD8<sup>+</sup> T cells were subsequently cocultured with dendritic cell/allogeneic COLM-6 fusion cells at a ratio of 10:1 in the presence of 20 units/mL human IL-2. After 7 days culture, cells were cultured in 96-well U-bottomed culture plates at indicated numbers per well. For monoclonal antibody (mAb) blocking assays, dendritic cell/COLM-6 allogeneic fusion cells were cocultured with whole T cells, purified CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells in the presence of mAbs against MHC class I (W6/32, 5 µg/mL) and MHC class II (HLA-DR, 5 µg/mL), MHC class II, or MHC class I, respectively before the onset of cultures and subsequently during the entire culture period. Dye solution was added to each well and incubated for 4 hours according to the protocol of Cell Titer 96 nonradioactive cell proliferation assay Kit (Promega). The measurement was done in Microplate Imaging System (Bio-Rad, Hercules, CA) at an absorbance of 630 nm.

**IFN- $\gamma$ , interleukin-2, interleukin-4, and interleukin-10 production.** To determine the stimulation of T cells by dendritic cell/tumor fusion cells, intracellular staining of IFN- $\gamma$ , IL-2, IL-4, and IL-10 was done.

<sup>7</sup> E. Hara, H. Sakamoto, Hashiguchi, et al., manuscript in preparation.

**Table 1.** Summary of clinical data from patients with colorectal cancer

Patient list	Colorectal cancer	HLA-ABC	HLA-DR	HLA-A2	HLA-A24	CEA	MUC1
1	Liver metastasis	+	–	+	+	+	+
2	Liver metastasis	+	–	–	+	+	+
3	Liver metastasis	+	–	–	+	+	+
4	Lung metastasis	+	–	+	+	+	+
5	Liver metastasis	+	–	+	–	+	+
6	Primary	+	–	+	+	+	+
7	Lymph node metastasis	+	–	+	+	+	+
8	Primary	+	–	–	–	+	+

Nonadherent PBMCs were cocultured with dendritic cell/tumor fusion cells, dendritic cells mixed with tumor cells, dendritic cells alone, or tumor cells alone at a ratio of 10:1 in the complete RPMI 1640 for 7 to 10 days and then harvested by nylon wool separation for analysis of human IFN- $\gamma$ , IL-2, IL-4, and IL-10 production using each cytokine secretion assay kit according to manufacturer's instructions (Miltenyi Biotec). Briefly, T cells were washed with cold PBS, incubated with cytokine Catching Reagent for 5 minutes at 4°C, 10 mL of prewarmed complete medium were added with shaking, and cultured for 45 minutes at 37°C. After incubation, cells were washed with cold PBS and stained with phycoerythrin-conjugated each cytokine detection antibodies and then incubated with FITC-labeled CD4 or CD8 mAb (Miltenyi Biotec) for 20 minutes on ice. Cells were washed, fixed, and analyzed by FACScan (BD Biosciences, San Jose, CA).

**Pentameric assay.** Complexes of phycoerythrin-labeled HLA-A2-MUC1 pentamer (950-958, STAPPVHNV), HLA-A2-CEA pentamer (571-579, YLSGANLNL), or HLA-A24-CEA pentamer (652-660, TYACFVSNL) or irrelevant pentamer were used (PROIMMUNE, Oxford, United Kingdom) to detect antigen-specific CTL induced by dendritic cell/autologous colorectal carcinoma or dendritic cell/allogeneic COLM-6 fusion cells. The pentameric staining was done according to the manufacturer's instructions. Briefly, the nonadherent PBMCs were cocultured with dendritic cells, tumor cells, dendritic cells mixed with tumor cells, or dendritic cell/tumor fusion cells for 7 to 10 days. Then, the purified T cells were incubated with phycoerythrin-conjugated pentamer for 10 to 15 minutes at room temperature in the dark. After washing with PBS, FITC-conjugated anti-human CD8 mAb was incubated for 20 to 30 minutes at 4°C in the dark. Cells were washed, fixed, and analyzed by FACScan using CellQuest analysis software (BD Biosciences).

**Cytotoxicity assays.** Nonadherent PBMCs were stimulated with dendritic cell/tumor fusion cells in the presence of 20 units/mL human IL-2. Nonadherent PBMCs cocultured with dendritic cells mixed with tumor cells, dendritic cells, or tumor cells alone were used as a control. The cytotoxicity assays were done by flow cytometry CTL assays using Active Caspase-3 Apoptosis kit I (BD PharMingen; refs. 26, 27). T2 cells were pulsed overnight with 10  $\mu$ g/mL of HLA-A2-MUC1 peptide (STAPPVHNV), HLA-A2-CEA peptide (YLSGANLNL), or control HLA-A2-influenza matrix peptide (GILGFVFTL; PROIMMUNE). The target cells, including autologous colorectal carcinoma cells, allogeneic tumor cell lines, autologous monocytes, T2 cells pulsed with peptides, and natural killer-sensitive K562 cells, were labeled with the red fluorescence dye PKH-26 (Sigma, St. Louis, MO). After washing with PBS, PKH-26-labeled target cells were cultured with T cells for 2 hours at 37°C in 96-well, V-bottomed plates. In certain experiments, PKH-26-labeled target cells were preincubated with anti MHC class I (mAb W6/32; 1:100 dilution), anti-HLA-A2 or anti-HLA-A24 mAb (1:100 dilution), or control IgG for 30 minutes at 37°C before addition of effector cells. Cells were washed, fixed with Cytofix/Cytoperm Solution (BD PharMingen), and then washed with Perm/Wash

Buffer (BD PharMingen). Cells were incubated with FITC-anti-human active caspase-3 substrate (BD PharMingen) for 30 minutes at room temperature followed by two washes with Perm/Wash Buffer. The percentage of caspase-3-positive cells in PKH-26-labeled target cell population was determined by the following calculation: % caspase-3 staining = [(caspase-3<sup>+</sup>PKH-26<sup>+</sup> cells) / (caspase-3<sup>+</sup>PKH-26<sup>+</sup> + caspase-3<sup>–</sup>PKH-26<sup>+</sup>)]  $\times$  100.

**Cold-target inhibition assays.** Cold target inhibition assays were done to investigate the target specificity of CTL induced by dendritic cell/allogeneic COLM-6 fusion cells. Briefly, the cytotoxic activities against PKH-26-labeled target cells, autologous colorectal carcinoma cells and COLM-6 were determined at the a ratio of 60:1 or 30:1 in the presence of 20-fold excess of PKH-26-unlabeled competitor cells, COLM-6 and autologous colorectal carcinoma cells, respectively. Percentage of cytotoxicity (mean  $\pm$  SD of three replicates) was determined by flow cytometry CTL assay.

## Results

**Characterization of dendritic cell/allogeneic COLM-6 fusion cells.** Monocyte-derived autologous immature dendritic cells were generated from patients with colorectal carcinoma (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) in the presence of granulocyte macrophage colony-stimulating factor and IL-4 for 7 to 10 days. The immature dendritic cells from patient 1 displayed a characteristic phenotype with expression of MHC class I (with HLA-A2 and HLA-A24 molecules), MHC class II, and costimulatory molecules (B7-1 and B7-2) and low levels of MUC1 but not CEA (Fig. 1A). By contrast, colorectal carcinoma cells isolated from patients expressed high levels of CEA, MUC1, MHC class I, HLA-A2/HLA-A24, but not MHC class II, B7-1 and B7-2 molecules (Fig. 1A; Table 1). The allogeneic colorectal carcinoma cell line, COLM-6, expressed high levels of CEA, MUC1, MHC class I, but not HLA-A2/HLA-A24, MHC class II, and B7-1 and B7-2 molecules (Fig. 1A). Fusion of allogeneic COLM-6 to autologous dendritic cells resulted in the generation of heterokaryons that express the CEA and MUC1 antigens, MHC class I (with HLA-A2 and HLA-A24 molecules), MHC class II, and B7-2 molecules, similar to that obtained with dendritic cell/autologous colorectal carcinoma fusion cells (Fig. 1A and B). Fusion efficiency determined by two-color flow cytometry showed that about 33.28  $\pm$  8.46% of dendritic cell/autologous colorectal carcinoma fusion cells and 34.50  $\pm$  12.26% of dendritic cell/allogeneic COLM-6 fusion cells dual expressed both CEA and HLA-DR (Fig. 1B; Table 2).

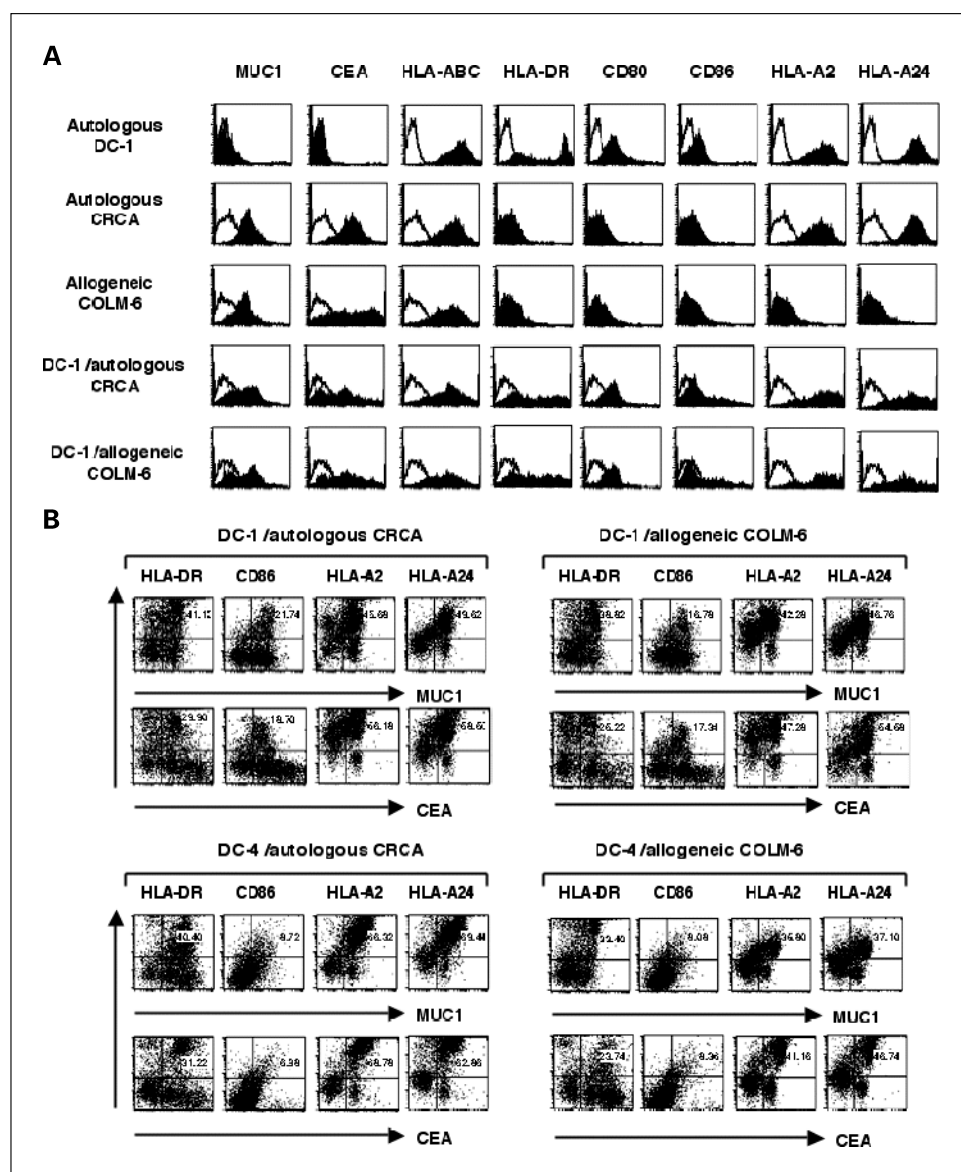


Fig. 1. Phenotype of autologous colorectal carcinoma cells or allogeneic COLM-6 cells fused to immature autologous dendritic cells. *A*, autologous dendritic cells from patient 1, autologous colorectal carcinoma (CRCA) cells, allogeneic COLM-6 cells, dendritic cell (DC)/autologous colorectal carcinoma fusion cells, and dendritic cell/allogeneic COLM-6 fusion cells were analyzed by flow cytometry for expression of the indicated antigens (solid area). *B*, dendritic cell/autologous colorectal carcinoma fusion cells or dendritic cell/allogeneic COLM-6 fusion cells from two patients (patients 1 and 4) were analyzed by two-color flow cytometry for expression of CEA/MUC1 and HLA-DR, CD86, HLA-A2, or HLA-A24.

**Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by dendritic cell/allogeneic COLM-6 fusion cells.** To assess the stimulating ability of dendritic cell/allogeneic COLM-6 fusion cells, autologous T cells were cocultured with dendritic cell/allogeneic COLM-6 fusion cells or dendritic cell/autologous colorectal carcinoma fusion cells for 3 days. Dendritic cell/allogeneic COLM-6 fusion cells but not dendritic cells mixed with allogeneic COLM-6 cells had effect on proliferation of autologous T cells (Fig. 2A). The results also show that the proliferation of T cells stimulated with dendritic cell/allogeneic COLM-6 fusion cells is similar to that obtained with dendritic cell/autologous colorectal carcinoma fusion cells. Moreover, dendritic cells from patient 2/allogeneic COLM-6 fusion cells induced the proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Fig. 2B, top). In antibody blocking experiments, the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was strongly inhibited by the presence of mAb against MHC class II and I molecules, respectively (Fig. 2B, bottom). These

results show that dendritic cell/allogeneic COLM-6 fusion cells initiate both MHC class I and II restrictive T-cell proliferation.

**Cytokine production of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by dendritic cell/allogeneic COLM-6 fusion cells.** To determine the T-cell activation by dendritic cell/allogeneic COLM-6 fusion cells, we used the cytometric cytokine production assay. Nonadherent PBMCs were cocultured with dendritic cell/allogeneic COLM-6 fusion cells or dendritic cell/autologous colorectal carcinoma fusion cells for 6 to 10 days, and T cells were purified by nylon wool separation. A high level of IFN- $\gamma$  production and, to a lesser extent, IL-10 production was detected in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated by dendritic cell/allogeneic COLM-6 fusion cells as well as dendritic cell/autologous colorectal carcinoma fusion cells. The specificity of T-cell responses to dendritic cell/allogeneic COLM-6 fusion cells was confirmed by the lack of IFN- $\gamma$  production from T cells stimulated by dendritic cells mixed with COLM-6 (data not shown). Minimal

**Table 2.** Percentage of dendritic cell/tumor fused cells

Patient list	Dendritic cell/autologous colorectal carcinoma (%)	Dendritic cell/allogeneic COLM-6 (%)
1	29.90	25.22
2	33.94	35.94
3	27.71	37.28
4	31.22	23.74
5	48.74	57.89
6	28.19	38.28
7	23.54	23.16
8	42.98	62.42
Mean $\pm$ SD	33.28 $\pm$ 8.46	34.50 $\pm$ 12.26

NOTE. %, percentage of cells positive for CEA and HLA-DR.

production of IL-2 and IL-4 was detected in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in this study (Fig. 3). These results indicate that dendritic cell/allogeneic COLM-6 fusion cells as well as dendritic cell/autologous colorectal carcinoma fusion cells activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

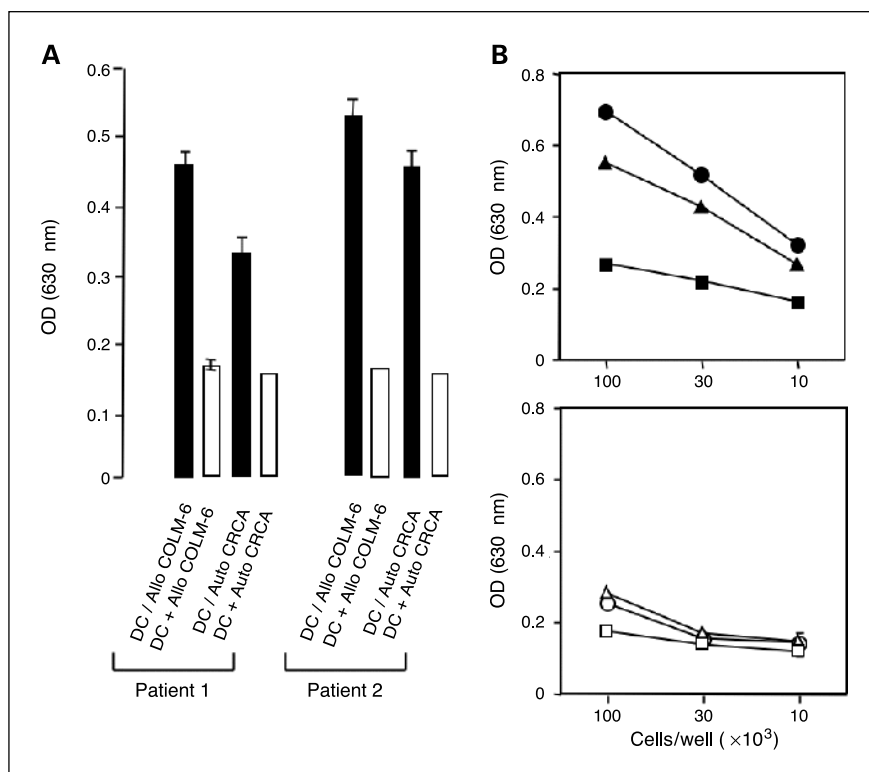
**Induction of CTL against autologous colorectal carcinoma cells by dendritic cell/allogeneic COLM-6 fusion cells.** The cytotoxicity assays were done by flow cytometry CTL assay that was predicated on measurement of CTL-induced caspase-3 activation in target cells through detection of the specific cleavage of fluorogenic caspase-3 (26, 27). First, we

examined whether dendritic cells from a healthy volunteer (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) fused with allogeneic COLM-6 (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) were able to elicit T cells with cytotoxic activity against COLM-6 used as a fusion. As shown in Fig. 4A, dendritic cell/allogeneic COLM-6 fusion cells can stimulate naive T cells and induced CTL that were able to lyse not only COLM-6 as a fusion partner but also allogeneic tumor cell lines positive for HLA-A2<sup>+</sup> and/or HLA-A24<sup>+</sup>, CEA, and/or MUC1. By contrast, dendritic cells mixed with allogeneic COLM-6 did not induce CTL responses. Moreover, preincubation of the target cells with an anti-HLA-ABC mAb inhibited the lysis, indicating restriction by MHC class I.

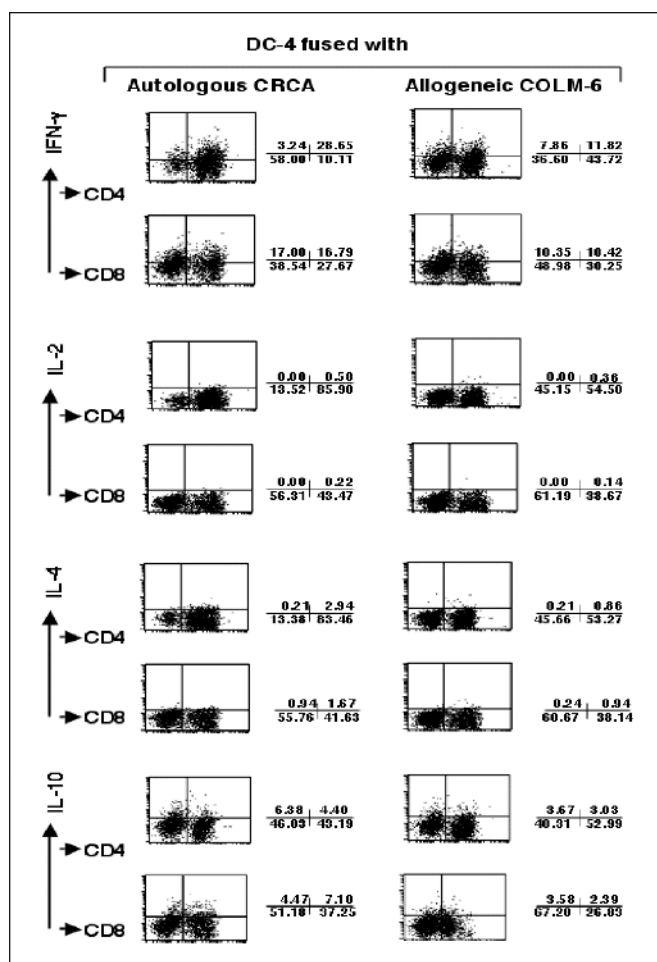
The next question is whether the fusion cells created by autologous dendritic cells from patients and allogeneic COLM-6 cells can induce CTL with cytotoxic activity against autologous colorectal carcinoma cells. In all the cases examined, T cells stimulated with dendritic cell/allogeneic COLM-6 fusion cells were effective in inducing significant lysis of autologous colorectal carcinoma cells. Similar results were obtained with T cells stimulated by dendritic cell/autologous colorectal carcinoma fusion cells (Fig. 4B). By contrast, T cells that had been cocultured with dendritic cells, tumor cells, or an unfused mixture of both failed to exhibit significant lysis of autologous colorectal carcinoma cells. Moreover, CTL activity was inhibited by preincubation of autologous colorectal carcinoma cells with anti-HLA-ABC mAb, indicating restriction by HLA class I (Fig. 4C).

**Recognition of HLA-A2 and/or HLA-A24 restricted tumor-associated antigen-specific targets by CTL induced with dendritic cell/allogeneic COLM-6 fusion cells.** To assess the colorectal carcinoma antigen specificity and HLA restriction element of

**Fig. 2.** Proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by dendritic cell/allogeneic COLM-6 fusion cells. *A*, nonadherent PBMCs from the two patients (patients 1 and 2) were cocultured with dendritic cell (DC)/allogeneic COLM-6 fusion cells or dendritic cell/autologous colorectal carcinoma fusion cells (black columns) at a ratio of 10:1 for 7 days in the presence of human IL-2 (20 units/mL). As a control, nonadherent PBMCs were also stimulated by dendritic cells mixed with allogeneic COLM-6 or autologous colorectal carcinoma cells (white columns). T-cell proliferation was done by Cell Titer 96 nonradioactive cell proliferation assay kit according to the protocol. *B*, whole T cells (●), purified CD4<sup>+</sup> T cells (▲), or purified CD8<sup>+</sup> T cells (■) from patient 2 were subsequently cocultured with dendritic cell/allogeneic COLM-6 fusion cells at a ratio of 10:1 for 7 days in the presence of human IL-2 (20 units/mL). T-cell proliferation was done in indicated numbers of T cells (top). For mAb blocking assays, mAb against MHC classes I and II was added to whole T cells (○); MHC class II mAb was added to purified CD4<sup>+</sup> T cells (□); or MHC class I mAb was added to purified CD8<sup>+</sup> T cells (□) before the onset of cultures and subsequently during the entire culture period (bottom). Similar results were obtained from T cells stimulated by dendritic cell/autologous colorectal carcinoma fusion cells in three independent experiments.



CTL induced by dendritic cell/allogeneic COLM-6 fusion cells, we used CTL assay using autologous colorectal carcinoma and multiple allogeneic tumor cell lines as targets in the same set of experiments, and T cells stimulated by dendritic cell/allogeneic Jurkat fusion cells that do not express colorectal carcinoma antigens are used as a control. As shown in Fig. 4B and Fig. 5A and B, CTL induced by autologous dendritic cells (HLA-A2<sup>+</sup> and/or HLA-A24<sup>+</sup>) fused with allogeneic COLM-6 (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) lysed not only autologous colorectal carcinoma cells (HLA-A2<sup>+</sup> and/or HLA-A24<sup>+</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) but also HLA class I-semimatched colorectal carcinoma cell lines COLP-2 (HLA-A2<sup>+</sup>), COLM-7 (HLA-A2<sup>+</sup>), and COLP-12 (HLA-A24<sup>+</sup>); breast cancer cell line MCF-7 (HLA-A2<sup>+</sup>); and ovarian cancer cell line OVAP-1 (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>), endogenously expressing CEA and/or MUC1, suggesting the additive effect of antigen-specific CTL. In addition, lysis of the targets was abrogated by preincubation of the tumor cells with anti-HLA-ABC mAb, indicating restriction by MHC class I



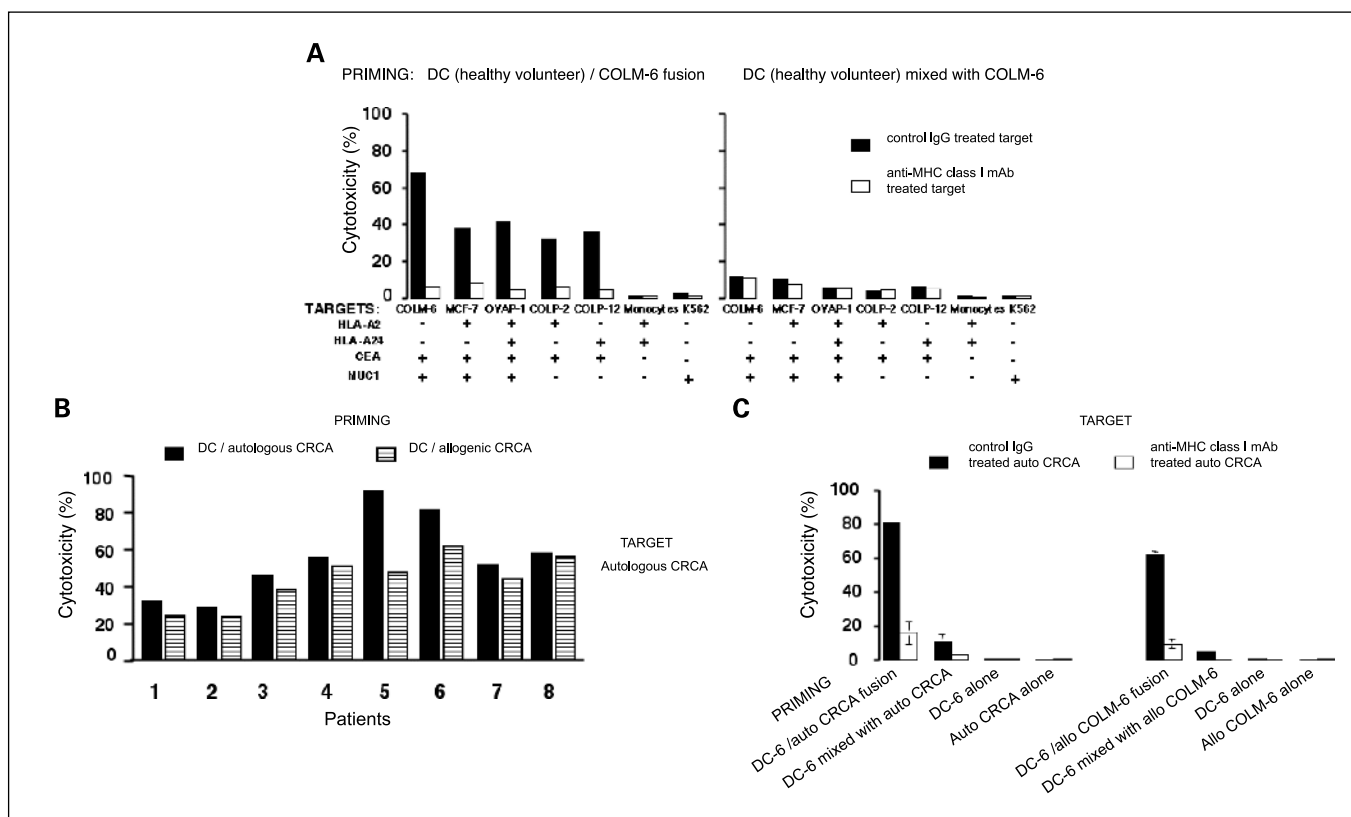
**Fig. 3.** Cytokine production in CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated by dendritic cell/allogeneic COLM-6 fusion cells. A, nonadherent PBMCs from patient 4 were incubated either with dendritic cell (DC)/autologous colorectal carcinoma (CRCA) fusion cells or dendritic cell/allogeneic COLM-6 fusion cells in the presence of human IL-2 (20 units/mL) for 6 days. Autologous T cells were stained with FITC-anti-CD4 and FITC-anti-CD8 mAb assessed for the cytokine production using human IFN-γ, IL-2, IL-4, and IL-10 production assay kits according to the manufacturer's protocol. Similar results were obtained in individual experiments using T cells derived from all different patients stimulated by dendritic cell/allogeneic COLM-6 fusion cells.

(Fig. 5B). By contrast, T cells stimulated by dendritic cell (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>)/autologous colorectal carcinoma fusion cells showed no lysis of COLM-6, confirming that HLA-A2 and/or HLA-A24 were the restriction element of CTL (Fig. 5A and B). Moreover, T cells stimulated by dendritic cell/allogeneic Jurkat fusion cells that did not express colorectal carcinoma antigens (CEA and MUC1) were not able to lyse autologous colorectal carcinoma cells, indicating that dendritic cell fused to allogeneic colorectal carcinoma cell line with shared colorectal carcinoma antigens (CEA and/or MUC1) induced colorectal carcinoma-specific CTL responses (Fig. 5C).

**Recognition of HLA-A2 and/or HLA-A24 restricted carcinoembryonic antigen and MUC1 peptides and allogeneic HLA class I molecules by CTL induced with dendritic cell/allogeneic COLM-6 fusion cells.** To analyze the CEA and/or MUC1 specificity and HLA restriction element of CTL induced by dendritic cell/allogeneic COLM-6 fusion cells, we used three assays: (a) CTL assay using T2 cell pulsed with CEA or MUC1 peptides; (b) pentameric assay specific for HLA-A2 CEA, HLA-A2 MUC1, and HLA-A24 CEA peptides; and (c) cold-target inhibition assay. Autologous T cells stimulated by dendritic cell/allogeneic COLM-6 fusion cells lysed T2 cells pulsed with CEA or MUC1 peptides but not T2 cells pulsed with control influenza matrix peptides, indicating CEA- and MUC1-specific and HLA-A2 restriction (Fig. 6A). In addition, lysis of the targets was abrogated by preincubation of the tumor cells with anti-HLA-ABC, HLA-A2, or HLA-A24 mAb, indicating restriction by HLA-A2 and HLA-A24 (Fig. 6A). As shown in Fig. 6B, CEA- and MUC1-specific CTL were detected in T cells stimulated with dendritic cell/allogeneic COLM-6 fusion cells, similar to that obtained with dendritic cell/autologous colorectal carcinoma fusion cells. In contrast, no positive T cells were detected when an irrelevant pentamer was used (data not shown). Moreover, CEA- and MUC1-specific CTL were not detected in T cells stimulated by the unfused mixture of dendritic cells and tumor cells (data not shown). Taken together, these results indicate that dendritic cell/allogeneic COLM-6 fusion cells can present colorectal carcinoma peptides and induce CEA- and MUC1-specific CTL in HLA-A2 and/or HLA-A24 restriction mechanisms. To further analyze the target specificity of CTL, we did cold-target inhibition assays. As shown in Fig. 6C, CTL activity against autologous colorectal carcinoma cells and COLM-6 was not influenced in the presence of 20-fold excess of COLM-6 and autologous colorectal carcinoma cells, respectively, emphasizing that CTL induced by dendritic cell/allogeneic COLM-6 fusion cells recognize both shared TAAs and mismatched MHC class I molecules.

## Discussion

The present studies describe an alternative approach to a dendritic cell/tumor fusion cell vaccine for patients with colorectal carcinoma. The fusion of dendritic cells with autologous tumor cells is an effective approach for introducing both known and unidentified TAAs into dendritic cells to induce polyclonal CTL responses (16–19, 28). The major limiting factor for the development of this approach for clinical use is the availability of adequate amounts of tumor cells. Especially, it is difficult to obtain sufficient numbers of viable



**Fig. 4.** Induction of CTL responses against autologous colorectal carcinoma cells by dendritic cell/allogeneic COLM-6 fusion cells. *A*, nonadherent PBMCs from a healthy volunteer (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) were cocultured with dendritic cell (DC)/allogeneic COLM-6 fusion cells (left) or dendritic cell mixed with allogeneic COLM-6 (right) for 7 to 10 days. T cells were cocultured with control IgG-pretreated COLM-6, MCF-7, OVAP-1, COLP-2, COLP-12, autologous monocytes, or K562 at a ratio of 60:1. For mAb block experiment, the stimulated T cells were cocultured with MHC class I mAb (W6/32; 1:100 dilution) pretreated target cells at a ratio of 60:1. *B*, nonadherent PBMCs obtained from eight different patients were stimulated by dendritic cell/autologous colorectal carcinoma (CRCA) fusion cells (black columns) or dendritic cell/allogeneic COLM-6 fusion cells (striped columns) for 7 to 10 days. T cells were purified by nylon wool and incubated with PKH-26-labeled autologous colorectal carcinoma cells at a ratio of 60:1. *C*, nonadherent PBMCs from patient 6 were cocultured with dendritic cell/tumor fusion cells, dendritic cell mixed with tumor, dendritic cell alone, or tumor cells alone for 10 days. T cells were purified by nylon wool and incubated with PKH-26-labeled autologous colorectal carcinoma cells at a ratio of 60:1. Autologous colorectal carcinoma cells were preincubated with control IgG (black columns) or anti-MHC class I mAb (W6/32; 1:100 dilution; white columns). Columns, mean percentage cytotoxicity of three replicates was determined by flow cytometry CTL assay; bars, SD.

colorectal carcinoma cells from primary lesion due to the length of culture time and potential contamination of bacteria and fungus. Moreover, the success rate of generating colorectal carcinoma cell lines from primary lesion is extremely low (about 10%).<sup>7</sup>

Our study is the first demonstration that autologous dendritic cells fused with allogeneic colorectal carcinoma cell line can prime T cells from patients to differentiate into antigen-specific CTL able to kill autologous colorectal carcinoma cells. This provides the experimental basis for using these allogeneic tumor cell lines as a fusion cell-based vaccine in the treatment of patients with colorectal carcinoma. Autologous dendritic cells (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) have been fused with autologous colorectal carcinoma cells or allogeneic COLM-6 cell line (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>) to generate dendritic cell/tumor fusion cells. The phenotype and function of the autologous dendritic cells fused to allogeneic COLM-6 cells was similar to that obtained with autologous colorectal carcinoma. Most importantly, dendritic cell/allogeneic COLM-6 fusion cells can induce CTL activity against autologous colorectal carcinoma cells and several HLA-A2<sup>+</sup> and/or HLA-A24<sup>+</sup> targets with shared TAAs, similar to that obtained with dendritic cell/autologous

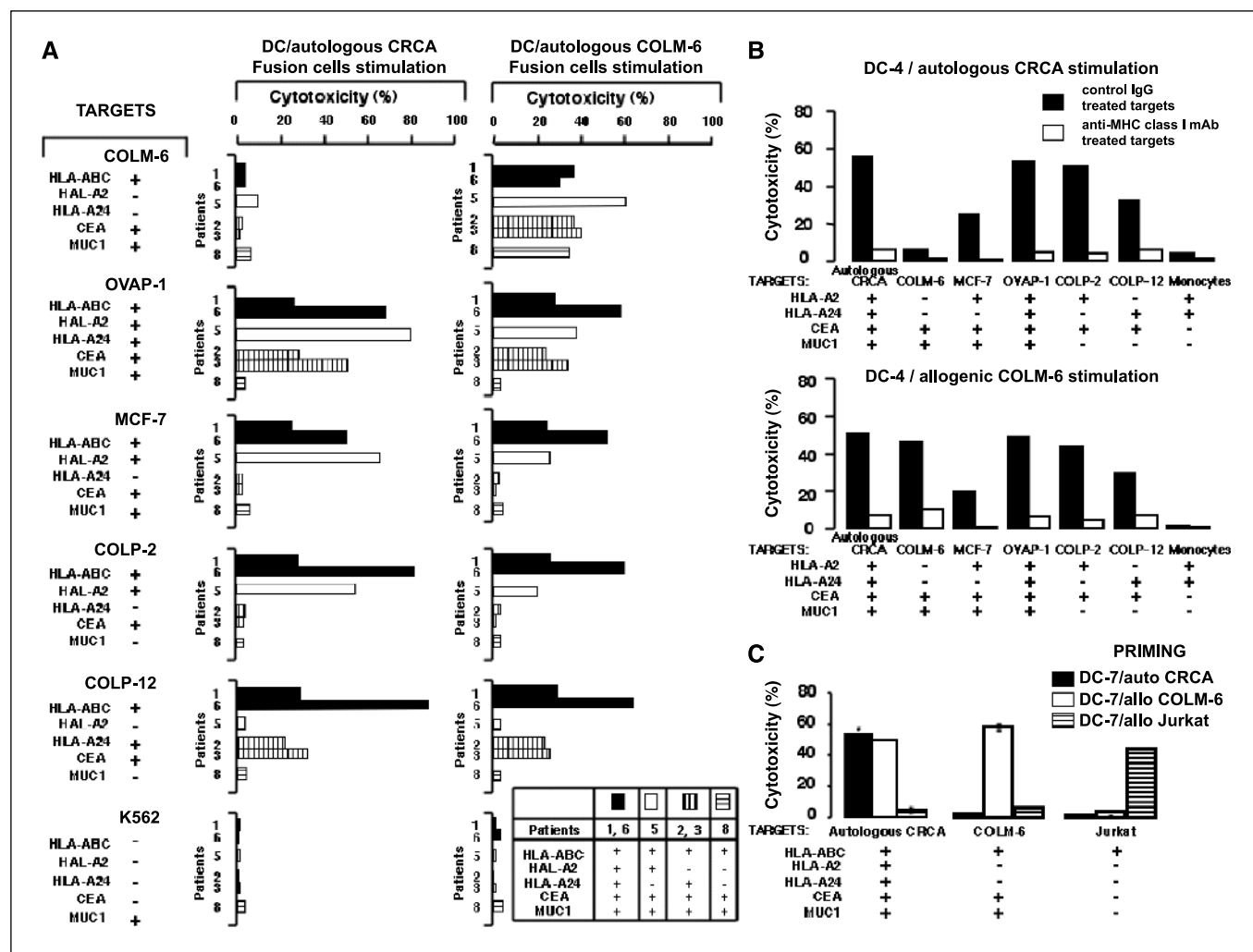
colorectal carcinoma fusion cells. Moreover, dendritic cell/allogeneic COLM-6 fusion cells were also able to stimulate alloreactive T cells probably due to the presence of allogeneic HLA class I molecules from allogeneic COLM-6 cells as a fusion partner. Thus, alloreactive T cells release cytokines, which may contribute to enhance and/or maintain the colorectal carcinoma-specific CTL responses. Importantly, allogeneic MHC class I molecules did not prevent the development of CTL specific for shared TAAs. Thus, allogeneic tumor cell lines as a source of TAAs can be used to elicit autologous colorectal carcinoma-specific CTL from the patients with colorectal carcinoma through the cross-priming against shared TAAs. A recent study that whole allogeneic tumor vaccine in hormone-resistant prostate cancer improved the nature of the disease (29) supports our findings that allogeneic tumor vaccine could have potential applicability to the field of antitumor immunotherapy. These findings in this study extend recent reports that fusion cells generated by autologous dendritic cells and autologous tumor cells induce antigen-specific CTL responses (18, 28).

Dendritic cell/tumor fusion cells have the ability to stimulate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, which are involved in the

antitumor immunity in the murine and human studies (19, 30, 31). High levels of IFN- $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated by dendritic cell/allogeneic COLM-6 fusion cells further suggests antigen presentation by fusion cell through both MHC class I and class II pathways simultaneously. The activation of CD4<sup>+</sup> T cells is significant because they play an important role in the induction and maintenance of CTL, and CD4<sup>+</sup> T cells have effector function against MHC class II-positive tumors (19, 30). The cytokine production patterns reflect the induction of polyclonal populations of activated T cells by dendritic cell/tumor fusion cells rather than the cytokine profile of an individual T cell (19, 32, 33). The low levels of IL-10 production did not seem to impair the induction of CTL responses in this study. In fact, there are

some reports suggesting that IL-10 stimulates activated CD8<sup>+</sup> T cells to proliferate and that it supports memory type of CD8<sup>+</sup> T-cell effectors capable of activation in an antigen-specific manner (34, 35). Human dendritic cell/allogeneic tumor fusion cells thus could be feasible tool for generating populations of tumor-reactive polyclonal CTL for use in adoptive immunotherapy.

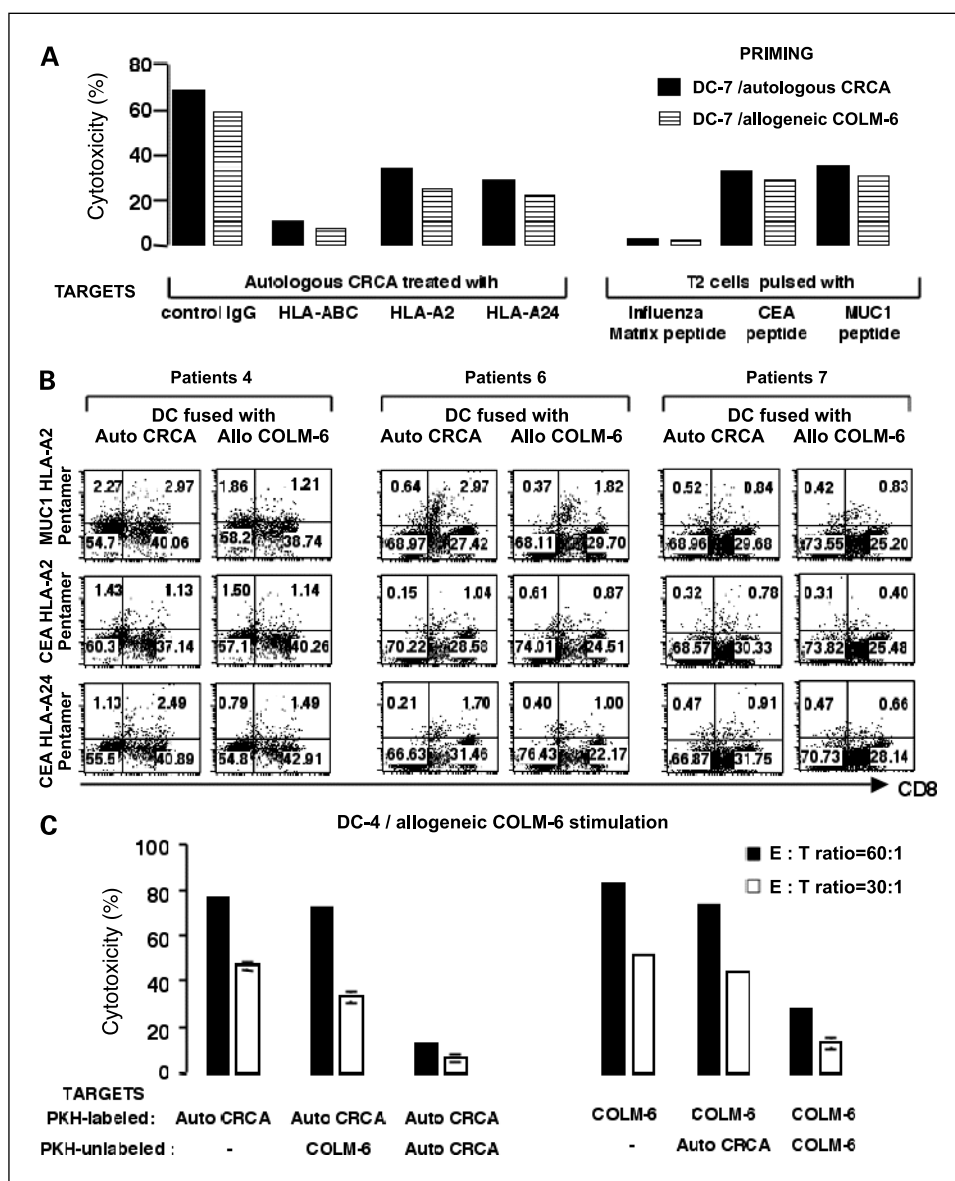
In summary, fusion cells generated by autologous dendritic cells and allogeneic colorectal carcinoma cells can induce antigen-specific CTL with activity against autologous colorectal carcinoma cells. This strategy have numerous advantages. (a) Allogeneic tumor cell lines are well characterized as TAA source. (b) Allogeneic tumor cell lines, which shared with TAAs, can grow well *in vitro*; thus, there is no limiting factor for



**Fig. 5.** Induction of shared colorectal carcinoma antigen-specific CTL restricted in HLA-A2 and/or HLA-A24 by dendritic cell/allogeneic COLM-6 fusion cells. *A*, nonadherent PBMCs from six patients (patients 1, 2, 3, 5, 6, or 8) stimulated by dendritic cell (DC)/autologous colorectal carcinoma (CRCA) fusion cells (left) or dendritic cell/allogeneic COLM-6 fusion cells (right) were incubated with PKH-26-labeled allogeneic colorectal carcinoma cell lines (COLM-6, COLP-2, or COLP-12), breast cancer cell line (MCF-7), ovarian cancer cell line (OVAP-1), or K562 at a ratio of 60:1. *B*, nonadherent PBMCs (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) from patient 4 were stimulated by dendritic cell/autologous colorectal carcinoma (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) fusion cells (top) or dendritic cell/allogeneic COLM-6 (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) fusion cells (bottom) for 10 days. T cells were incubated with PKH-labeled autologous colorectal carcinoma cells, allogeneic colorectal carcinoma cell lines (COLM-6, COLP-2, or COLP-12), breast cancer cell line (MCF-7), ovarian cancer cell line (OVAP-1), or autologous monocytes at a ratio of 60:1. The target cells were preincubated with control IgG (black columns) or anti-MHC class I mAb (W6/32; 1:100 dilution; white columns). *C*, nonadherent PBMCs (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) from patient 7 were stimulated by dendritic cell/autologous colorectal carcinoma (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) fusion cells, dendritic cell/allogeneic COLM-6 (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>, CEA<sup>+</sup>, and MUC1<sup>+</sup>) fusion cells, or dendritic cell/allogeneic Jurkat (HLA-A2<sup>-</sup>/HLA-A24<sup>-</sup>, CEA<sup>-</sup>, and MUC1<sup>-</sup>) fusion cells for 10 days. T cells were incubated with PKH-labeled target cells at a ratio of 60:1. Columns, mean percentage cytotoxicity of three replicates was determined by flow cytometry CTL assay; bars, SD.



**Fig. 6.** Induction of CEA- and MUC1-specific CTL and alloreactive T cells by dendritic cell/allogeneic COLM-6 fusion cells. **A**, nonadherent PBMCs (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) from patient 7 were stimulated by dendritic cell (DC)/autologous colorectal carcinoma (CRCA) fusion cells (black columns) or dendritic cell/allogeneic COLM-6 fusion cells (striped columns) for 10 days. T cells were incubated with PKH-26-labeled autologous colorectal carcinoma cells at a ratio of 60:1. The target cells were preincubated with control IgG, anti-MHC class I, anti-HLA-A2, or anti-HLA-A24 mAb (1:100 dilution; left). T cells were also incubated with T2 cells pulsed with influenza matrix peptide, CEA peptides, or MUC1 peptides at a ratio of 60:1 (right). **B**, nonadherent PBMCs (HLA-A2<sup>+</sup>/HLA-A24<sup>+</sup>) from three patients (patients 4, 6, or 7) were cultured with dendritic cell/autologous colorectal carcinoma fusion cells or dendritic cell/allogeneic COLM-6 fusion cells for 7 to 10 days. T cells were collected and examined by HLA-A2/MUC1, HLA-A2/CEA, or HLA-A24/CEA pentameric assay. **C**, to further assess the target specificity of CTL induced by dendritic cells from patient 4/allogeneic COLM-6 fusion cells, the cytotoxicity against PKH-26-labeled target cells (autologous colorectal carcinoma cells or COLM-6) were done in the presence of 20-fold excess of PKH-26 nonlabeled competitor cells (autologous colorectal carcinoma or COLM-6) at a ratio of 60:1 (black columns) and 30:1 (white columns). Columns, mean percentage cytotoxicity of three replicates was determined by flow cytometry CTL assay; bars, SD.



preparation of tumor cells. (c) It is not necessary to determine HLA typing of patients and allogeneic tumor cells as a partner of fusion cells, because autologous dendritic cells can process and present multiple TAAs from allogeneic tumor cells in the

context of MHC classes I and II. Taken together, these results indicate that a dendritic cell/allogeneic colorectal carcinoma fusion vaccine can be used in the immunotherapy of patients with colorectal carcinoma.

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